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## *PLASMODIUM VAUGHANI* NOVY AND MACNEAL, 1904, IN THE NEW HEBRIDES: WITH A NOTE ON THE OCCURRENCE OF *ELONGATUM*-TYPE EXOERYTHROCYTIC SCHIZOGONY IN THIS SPECIES<sup>1</sup>

MARSHALL LAIRD<sup>2</sup>

Department of Zoology, Victoria University College, Wellington, New Zealand

### INTRODUCTION

Little is known concerning avian haematozoa in the South Pacific. The opportunity was accordingly taken to make blood smears from as many birds as possible during a recent malaria reconnaissance in the New Hebrides. In addition to the parasite discussed hereunder, species of *Trypanosoma*, *Haemoproteus* and *Microfilaria* were discovered, but these will be dealt with in another account.

### MATERIALS AND METHODS

Only one species of avian malarial parasite was encountered during these studies. This was recorded from the heart blood of two subspecies of the Yellow White-eye, *Zosterops flavifrons* Gmelin, which as Mayr (1945) stated is perhaps the most numerous bird of the New Hebrides. Of three examples of subsp. *majuscula* Murphy and Mathews, 1929, shot near Anelgauhat, Aneityum, on August 5, 1952, two harbored *Plasmodium* (in association with *Trypanosoma* and *Microfilaria*); while of two examples of subsp. *brevicauda* Murphy and Mathews, 1929, shot on Aore Island on August 13, 1952, one was infected with *Plasmodium* (again in association with *Trypanosoma* and *Microfilaria*).

No morphological variation in any way attributable to host or locality differences could be discerned in the *Plasmodium* from the two sources. As none of the hosts of this parasite harbored *Haemoproteus*, the possibility of confusing gametocytes with those of the latter genus did not arise. In each instance three thin heart blood smears were made from the bird immediately following collection. These were fixed in methyl alcohol and stained with Giemsa during the evening of the same day, in order to avoid tropical deterioration. If *Plasmodium* was not recorded following a half-hour microscopic search of each of the three slides, using  $\times 8$  oculars and a Zeiss Oil 100 N. A. 1.30 achromatic objective, the bird was regarded as negative for the genus.

### MORPHOLOGICAL ACCOUNT (Pl. 1, figs. 1-32)

ERYTHROCYTIC STAGES (figs. 1-21); all measurements in microns.

*Trophozoites*.—Small, typical signet-ring stages (fig. 1) being rather rare. Most

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<sup>2</sup> Medical Branch, Royal New Zealand Air Force.

young trophozoites are pyriform (fig. 2) or of irregular outline. The diameter of the smallest one seen was 0.9, that of older and more massive ones (figs. 3, 4) ranging up to 2.7.

*Schizonts*.—Four (figs. 5, 6), six (figs. 7, 8) or eight (figs. 9, 10) nuclei are formed. The bulk of the segmenters are quadrinucleate. They appear cruciform, their nuclei being terminally and laterally located, and their cytoplasm sometimes stains quite a deep blue with Giemsa. Quadrinucleate segmenters range from 2.3 to 2.8 in length and from 1.6 to 2.0 in breadth, the nuclei ranging from 0.5 to 0.7 in diameter.

Hexa- and octonucleate segmenters are more or less ovoid. Their measurements at the greatest diameters range from 3.0 to 4.2 by 1.6 to 3.2. The nuclei are usually irregularly disposed, although they are occasionally grouped about the pigment in the form of a rosette (fig. 9). The cytoplasm of these larger segmenters seldom takes up any stain at all.

One (figs. 5, 10) or two (figs. 6–9) pigment granules are present. Where two such granules occur, one is always appreciably larger than the other, and sometimes this larger granule (fig. 8) or the single large one (fig. 10) is refringent. The pigment is of a black or brownish-black color.

*Merozoites*.—These appear as hollow masses of chromatin, no cytoplasm whatsoever being evident (figs. 11, 12). They range in length from 0.5 to 0.8.

*Gametocytes*.—The youngest stages identified (fig. 13) are elongate-ovoid, while at the intermediate stage of development long pseudopodia may be present (fig. 14). Mature gametocytes are elongate, and their outline is often decidedly irregular (figs. 16–21). Sometimes only from four (fig. 16) to six (fig. 20) large pigment granules are present, but macrogametocytes having up to 21 such granules, mostly of small size, have been noticed (fig. 15). Similarly, as many as 14 granules have been counted in microgametocytes (fig. 17).

The length of mature macrogametocytes ranges from 9.3 to 11.7, and the breadth from 1.2 to 2.1. Mature microgametocytes range from 8.9 to 11.7 in length, and from 1.7 to 2.5 in breadth. The cytoplasm of macrogametocytes stains pale blue with Giemsa, while that of microgametocytes appears whitish. Both kinds of gametocyte stain so feebly that it is often difficult to define the body limits of the parasite. Macrogametocytes have a round (fig. 18) or irregularly shaped (fig. 16) nucleus, which assumes a pale pink stain. The nucleus of microgametocytes is usually more or less saddle-shaped, and frequently stains at the periphery only (figs. 17, 19, 20).

#### EXOERYTHROCYTIC STAGES (figs. 22–28, 32)

*Trophozoites*.—These have been observed in basophil (fig. 22) and polychromatophil (fig. 24) erythroblasts. It is usually difficult to differentiate their cytoplasm from that of the host cell.

*Schizonts*.—Quadrinucleate examples have been seen in basophil (fig. 23) and polychromatophil (fig. 25) erythroblasts, also in small and large (fig. 27) lymphocytes. Fig. 26 shows a pigmented hexanucleate segmenter in a normoblast, while a large non-pigmented example in a circulating macrophage is illustrated in fig. 28. Those forms in leucocytes and basophil erythroblasts never have pigment, while non-pigmented schizonts have also been seen in some polychromatophil erythroblasts.

Exoerythrocytic schizonts are usually larger than the equivalent stages occurring in mature red cells, and their nuclei are larger than those of the latter stages. The length of quadrinucleate forms ranges from 2.6 to 3.4, and the breadth from 2.2 to 2.7; while hexanucleate segmenters measure from 4.2 to 5.1 by 3.4 to 4.2. The nuclei themselves average 1.0 in diameter.

*Merozoites*.—The only white cell in which separated and unaltered merozoites have been noticed is the myelocyte illustrated in fig. 32. Once again, these appear simply as ring-like masses of chromatin.

#### EFFECT OF THE PARASITE ON THE HOST CELL

Beyond occasional lateral (figs. 10, 21) or longitudinal (fig. 25) displacement of the nucleus, this *Plasmodium* causes no noticeable alteration of the host cell.

#### PATHOLOGY

None of the *Zosterops* examined were completely free from haematozoa, one of the examples uninfected with *Plasmodium* being positive for *Trypanosoma* and *Microfilaria* and the other for *Microfilaria* only. It is of interest that while erythroblasts made up but 5 per cent of the red cell total in the example of *Z. flavifrons majuscula* positive for *Trypanosoma* and *Microfilaria* only, these cells accounted for 15 per cent of the red cell total in one of the birds suffering from malaria in addition to the other two genera of parasites. Basophil erythroblasts were about ten times as abundant in the heart blood of the latter bird than in that of the former, and circulating macrophages were much more abundant. These facts suggest a considerable hyperplasia of the bone marrow in the malaria-infected bird, although controlled experiments would obviously be necessary for conclusive data on this point to be obtained.

#### PARASITE LEVEL

None of the three infections were particularly heavy ones. A random count of 10,000 mature erythrocytes from the bird displaying the highest parasitemia showed only 103 of these to be infected. Thirteen per cent of the infected cells contained trophozoites, 16 per cent contained gametocytes and 71 per cent contained schizonts. Seventy-four per cent of the schizonts were quadrinucleate, 17 per cent were hexanucleate, and 9 per cent were octonucleate.

#### SYSTEMATIC POSITION

Several of the known species of avian malarial parasites bear comparison with the *Plasmodium* under consideration. Hewitt (1949) summarized the morphological features of the following six small species, all of which have elongate gametocytes and characteristically form eight or less merozoites in each segmenter: *Plasmodium vauhani* Novy and MacNeal, 1904, *P. rouxi* Sergent, Sergent and Catanei, 1928, *P. nucleophilum* Manwell, 1935, *P. hexamerium* Huff, 1935, *P. oti* Wolfson, 1936, and *P. juxtannucleare* Versiani and Gomes, 1941. Manwell (1949) reduced this list of species to five, by demonstrating that *P. oti* is a synonym of *P. hexamerium*. Insofar as they are at present known, four of these species differ in detail from the New Hebridean one as outlined hereunder.

*P. rouxi* does not form more than four merozoites per segmenter; the gametocytes of *P. nucleophilum* are characteristically closely applied to the host-cell nucleus,

and mature segmenters are rarely seen in the peripheral blood; mature segmenters of *P. hexamerium* have clumped pigment and six or eight merozoites; while in *P. juxtannucleare* round as well as elongate gametocytes are formed, and mature segmenters contain from three to five merozoites.

Novy and MacNeal (1904) gave only brief information concerning *P. vaughani*, which was redescribed by Manwell (1935). Additional morphological data were presented by Garnham (1950). While commenting on the small size of the parasite, previous authors have published few actual measurements. The main features of *P. vaughani* may be summarized as follows.—

*Trophozoites*.—Small, often amoeboid (Manwell, Garnham).

*Schizonts*.—Usually four, but often six or eight merozoites (Manwell); nearly always four merozoites, very rarely six (Garnham). Quadrinucleate schizonts *Nuttallia*-like, about 2.5 long (Garnham). A single large pigment granule (Manwell, Garnham) or sometimes such a granule and one or even two small ones as well (Manwell). According to Manwell the large granule is generally very refractile, and of a brownish-black color; Garnham did not observe refringent granules. The mature schizont almost completely lacks cytoplasm (Garnham).

*Merozoites*.—The cytoplasm seems to disappear as the merozoites separate (Garnham). These structures have a densely staining peripheral ring of chromatic material, and are about 0.5 in diameter (Garnham).

*Gametocytes*.—Young gametocytes having pseudopodial processes are not infrequently seen (Manwell—that illustrated in his fig. 20 compares closely with the one shown in fig. 14 of the present paper). Mature examples elongate, there being little difference between macro- and microgametocytes apart from the lighter-staining cytoplasm of the latter (Manwell). Dimensions, 8 by 3 (Garnham). While Manwell's figures indicate that the pigment of these forms is present as four or five relatively large granules, Garnham's fig. 28 shows a total of about 24 smaller granules in two aggregations at opposite ends of the gametocyte.

*Exoerythrocytic stages*.—Not reported, although Hegner and Wolfson (1938a) searched for them in the reticulo-endothelial cells of canaries infected with two strains of *P. vaughani*. Hegner and Wolfson (1938) published data supplied by Manwell, referring to the occurrence of *Toxoplasma*-like bodies in the blood of canaries subinoculated with *P. vaughani* from a robin, although autopsy of the latter bird itself failed to reveal such bodies. Hewitt (1949) mentioned the tendency of *P. vaughani* to invade immature red cells.

*Effect of the parasite on the host cell*.—Usually no displacement of host-cell nucleus, and where this does occur it is only slight (Manwell).

*Infection rate*.—Four out of six robins examined by Manwell were parasitized; common in weavers, Kenya (Garnham).

*Parasite level*.—May be high in chronic period, but shows some variability (Manwell).

Manwell (1935) considered that *Plasmodium tenuis* (Laveran and Marullaz, 1914) is a synonym of *P. vaughani*. *P. tenuis* was described from the babbler, *Liothrix luteus*, which occurs in China and adjacent parts of the Orient. The type host was examined at Paris, and Wenyon recorded the species from an example of the same host at London (Sergeant, Sergeant and Catanei, 1931). Giovannola (1934) published a record of *P. tenuis* from the blackbird, *Turdus merula*, in Italy.

Manwell also thought it probable that the *tenuis*-like parasites described from two Formosan birds (*Prinia extensicauda* and *Trochalopteron taiwanum*) by Ogawa and Uegaki (1927), are referable to *P. vaughani*. Ogawa and Uegaki stated that young trophozoites were 1.5 in diameter, and that first two, then four or six and finally eight chromatin masses were formed in each schizont. The hexa- and octonucleate schizonts illustrated in their figs. 19–21 certainly resemble those of *P. vaughani* very closely. Uegaki (1930) briefly described a parasite resembling *P. tenuis* from the Japanese *Nycticorax nycticorax nycticorax*. While the octonucleate schizont illustrated in Uegaki's fig. 18 resembles the equivalent stage of *P. vaughani*, that author stated that although the characteristic number of merozoites formed was 4–8, as many as 20 were occasionally present. The possibility that Uegaki was dealing with a mixed infection must not be overlooked, but until this point has been investigated his parasite cannot be regarded as conspecific with *P. vaughani*.

Hewitt (1949) gave *Plasmodium tumbayaensis* (Mazza and Fiori, 1930), a parasite of the South American *Planesticus anthracinus*, as a second probable synonym of *P. vaughani*.

It is obvious that *P. vaughani*, *P. rouxi*, *P. nucleophilum*, *P. hexamerium* and *P. juxtannucleare* have close affinities with one another. More detailed morphological data concerning most of these species are still required. The range and average of the number of merozoites produced in each segmenter are usually the chief criteria for their morphological differentiation. Manwell (1949), in indicating that *P. oti* is a synonym of *P. hexamerium*, referred to the inadvisability of placing too great stress on these criteria, and pointed out that "in avian malaria at least, the average number of merozoites per segmenter falls considerably in the later stages of infection in some species." However, the New Hebridean *Plasmodium* described above compares most closely with that of the first member of the *vaughani* group to be designated; so closely, indeed, that it could only lead to confusion to regard it as new. It is therefore proposed to identify the parasite of *Zosterops flavifrons* as *Plasmodium vaughani*.

#### HOSTS AND DISTRIBUTION OF

##### *P. vaughani*

The type host is the North American robin, *Planesticus migratorius*. Manwell based his redescription of the parasite on material from the same host at Syracuse, New York. A starling, *Sturnus vulgaris vulgaris*, captured in Baltimore, Maryland, was subsequently found to be infected with a strain of *P. vaughani*, although morphological data were not published by the investigators concerned (Hegner and Wolfson, 1938a). The latter authors also indicated that canaries act as host for both the Manwell (2R) and Wolfson (2S) strains. If *P. tenuis* is a synonym of *P. vaughani*, as seems almost certainly to be the case, the host-list and range of the latter species will have to be extended. The same remarks apply in the case of *P. tumbayaensis*. Discounting the question of synonymy, however, the only previous record of *P. vaughani* from outside the U. S. A. is that of Garnham (1950), who reported it from weavers and fiscal shrikes in Kenya.

The present host record is also new. There is only one previous record of malarial parasites from a bird of the genus *Zosterops*. This concerns the Formosan

*Z. palpebrosa pegenis*, from which Ogawa and Uegaki (1927) described a single light infection. Although the species of *Plasmodium* involved was not determined, it obviously does not belong to the *vaughani* group. The illustrations published by Ogawa and Uegaki (their figs. 13-15) show that both schizonts and gametocytes cause distortion of the host cell and considerable displacement of the host-cell nucleus, and the parasite forms up to 18 merozoites per segmenter.

Garnham's record of *P. vaughani* from Kenya, together with the present one from the New Hebrides, justify Manwell's (1935) opinion that "It is probable that this species has a wide distribution".

INTRALEUCOCYTIC BODIES OF UNCERTAIN NATURE,  
ASSOCIATED WITH *P. vaughani*

In the heaviest infection noted, that from *Z. flavifrons majuscula* mentioned above under the headings "Pathology" and "Parasite Level", many lymphocytes and most of the circulating macrophages contained from one to many small inclusions staining a dense reddish-black with Giemsa. Illustrations of these bodies in small (fig. 29) and large (fig. 30) lymphocytes, and in a macrophage (fig. 31) are given in Pl. 1. The inclusions are of varying shape, some being dot-like, some irregularly rounded, and some more or less crescent-shaped. Sometimes they occur in pairs, but more often they are scattered through the cytoplasm of the cell concerned. They average 0.5 to 0.6 in diameter, the range being 0.1 to 1.5. In some instances the inclusions are surrounded by clear halos, while in others they are not.

Aragão (1911) and Herman (1937) published figures of irregularly-shaped intraleucocytic bodies from birds, closely resembling those under consideration. The former author believed his to represent the schizogonic stage of a haemogregarine, while Herman identified those he studied as *Toxoplasma*. Wolfson (1940) reclassified the *Toxoplasma*-like bodies known from birds into three types, I, II and III. She considered the inclusions figured by Aragón and Herman to belong to her Type I, which embraces numerous intraleucocytic forms now recognized as exoerythrocytic stages of *Plasmodium*.

The only *Toxoplasma*-like body previously recorded in association with *P. vaughani* was discovered by Manwell (Hegner and Wolfson, 1938), and Wolfson (1940) included it in her Type II. Organisms of this type are uninucleate, round or oval, and usually occur singly or in pairs. Where two are present within a leucocyte they are usually present one on either side of the host-cell nucleus, which is frequently deeply indented along the line of contact with the parasite. Type II *Toxoplasma*-like organisms are much larger than those of Type I. The generic name of *Atoxoplasma* was applied to them by Garnham (1950), with *A. avium* (Marullaz, 1913) as the type.

Wolfson's Type III organisms, which as she stated bear the closest morphological resemblance to mammalian *Toxoplasma*, were confirmed as belonging to this genus by Nobrega and Reis (1942) (according to Garnham, 1950).

It is obvious that the intraleucocytic bodies in question are not referable either to *Toxoplasma* or *Atoxoplasma*. Because of their irregular size, shape and number, and their structureless appearance, I would hesitate to regard them as plasmodial schizonts. As they were only observed in a preparation in which the schizogony of

*P. vaughani* was actively proceeding, it seems more feasible to regard them as phagocytosed and necrotic merozoites of this parasite.

## SUMMARY

*Zosterops flavifrons majuscula* from Aneityum, and *Z. flavifrons brevicauda* from Aore, are new host and locality records for *P. vaughani*. Exoerythrocytic stages of this parasite are described for the first time, trophozoites being recorded from basophil and polychromatophil erythroblasts, schizonts from these cells and also from normoblasts, lymphocytes and macrophages, and merozoites from a myelocyte. Intraleucocytic bodies of uncertain nature, tentatively regarded as phagocytosed and necrotic merozoites of *P. vaughani*, are also discussed.

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## EXPLANATION OF PLATE I

All figures prepared with the aid of a Zeiss-Winkel drawing apparatus at a magnification of  $\times 2,140$ .

*Plasmodium vaughani* Novy and MacNeal, 1904, from *Zosterops flavifrons* Gmelin subsp. *majuscula* Murphy and Mathews, 1929

- FIGS. 1-21. Erythrocytic stages.
- FIGS. 1-4. Trophozoites.
- FIGS. 5 and 6. Quadrinucleate segmenters.
- FIGS. 7 and 8. Hexanucleate segmenters.
- FIGS. 9 and 10. Octonucleate segmenters.
- FIGS. 11 and 12. Separated merozoites, not yet liberated from host cell.
- FIGS. 13 and 14. Young gametocytes.
- FIGS. 15, 16, 18. Macrogametocytes.
- FIGS. 17, 19-21. Microgametocytes.

## PLATE I



*Plasmodium vaughani* Novy and MacNeal, 1904

FIGS. 22-28, 32. Exoerythrocytic stages.

FIG. 22. Late trophozoite in basophil erythroblast.

FIG. 23. Quadrinucleate segmenter in basophil erythroblast.

FIG. 24. Ring form trophozoite in polychromatophil erythroblast.

FIG. 25. Quadrinucleate segmenter in polychromatophil erythroblast.

FIG. 26. Hexanucleate segmenter in normoblast.

FIG. 27. Quadrinucleate segmenter in large lymphocyte.

FIG. 28. Hexanucleate segmenter in circulating macrophage.

FIGS. 29-31. Phagocytosed merozoites (?) in a small lymphocyte (29), a large lymphocyte (30) and a circulating macrophage (31).

FIG. 32. Merozoites in myelocyte.

NORTH AMERICAN NEMATODES OF THE GENUS  
*PHARYNGODON* DIESING (OXYURIDAE)

CLARK P. READ<sup>1</sup> AND YOST U. AMREIN<sup>2</sup>

A perusal of the literature relating to the parasitic fauna of the reptiles in the arid southwestern United States indicates that little information has been published. This is perhaps surprising in view of the quantity of data available on the biology of the hosts. In a vast area extending west from the region of Texas studied by Harwood (1932) and south into the northern parts of Mexico very few parasitological studies have been carried out. There has been a recent awakening of interest in the nematode parasites of Southern California reptiles (Walton, 1941; Lucker, 1951; Read, Amrein, and Walton, 1952; Read and Amrein, 1952; Edgerly, 1952).

In continuing a survey of the parasites of reptiles in Southern California, the writers have found three new species of oxyurid nematodes belonging to the genus *Pharyngodon*. These and a fourth species of *Pharyngodon* from Cuba are described in the present paper. Appreciation is expressed to Mr. B. H. Brattstrom and Dr. A. C. Walton who made available a number of reptilian oxyurids, supplementing the collections made by the authors. The Cuban material was collected by the senior author.

The genus *Pharyngodon* Diesing is confined to reptilian and amphibian hosts. Of the thirty-one species undoubtedly belonging to the genus, five occur in amphibians; the remaining twenty-six species are parasites of lacertilians. Eight species have been reported from the Western Hemisphere.

Malan (1939) has furnished a valuable key to the species of *Pharyngodon*. Eleven species have been described since Malan's key was prepared. These are *P. kartana* Johnston and Mawson, *P. australis* Johnston and Mawson, *P. limnodynastes* Johnston and Mawson, *P. oxkutzcabiensis* Chitwood, *P. yucatanensis* Chitwood, *P. bassi* Walton, *P. polypedatis* Yamaguti, *P. papillocauda* Hannum, *P. geckinis* Liu and Wu, *P. neyrae* Calvente, *P. mearnsi* Edgerly, *P. medinae* Calvente, and *P. apapillosus* Koo. The two last named species are considered by the present authors to belong to the genus *Parathelandros* rather than to *Pharyngodon*. *P. australis* Johnston and Mawson may be specifically identical with *P. tiliquae* Baylis. These two species were described from the same host species and seem to differ mainly in size. The males of *P. limnodynastes* Johnston and Mawson, *P. batrachensis* Walton, and *P. armatus* Walton are unknown.

Calvente (1948) has proposed that the genus *Pharyngodon* be divided into two subgenera, *Pharyngodon* and *Neyrapharyngodon*. The sole character used by Calvente to separate these subgeneric groups is the presence or absence of a spicule. The writers are not convinced that this characteristic has sufficient evolutionary significance to be used meaningfully in the subgeneric partition of *Pharyngodon*. *P. cubensis*, to be described in the present paper, is obviously closely related to *P. auziensis* Seurat. These two species occur in congeneric hosts in Cuba and North

<sup>1</sup> Department of Zoology, University of California, Los Angeles

<sup>2</sup> Department of Biology, Pomona College

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Africa, respectively. *P. cubensis* lacks a spicule while *P. auziensis* is described as possessing a spicule. By Calvente's criterion these two species would be placed in different subgenera, creating a decidedly artificial taxonomic separation. There are several parallel instances in the genus. Thus, while it might be desirable from the standpoint of convenience to break up a genus that is becoming somewhat unwieldy, the writers do not consider Calvente's criterion for subgeneric grouping to be one which reflects the relationships of the species in the genus. The presence or absence of a spicule in the species of *Pharyngodon* constitutes a useful feature in constructing a key to the species but is probably of the same order of significance as the presence or absence of spines on the tail of the female.

*Pharyngodon giganticus* n. sp.

(Figs. 1-4)

**Diagnosis: Male:** 3.02 to 3.24 mm. long, maximum width 0.211 to 0.215 mm.; lateral alae about 0.053 mm. wide in posterior part of body, extending posteriorly from point 0.066 to 0.105 mm. behind anterior extremity; posterior limit of alae at level of anterior limit of caudal alae. Esophagus 0.314 to 0.360 mm. long; subglobular esophageal bulb 0.066 to 0.073 mm. long, 0.053 to 0.069 mm. wide. Excretory pore opens ventrally, 0.768 to 0.792 mm. from head. Well developed caudal alae present; each ala about 0.020 mm. wide and 0.060 mm. long. Three pairs of caudal papillae present; precloacal pair situated on somewhat inflated portion of caudal end; adcloacal pair somewhat postero-laterally directed; postcloacal papillae not enclosed by caudal alae. Prominent cloacal protuberance present. Cloacal opening 0.415 to 0.438 mm. from posterior extremity. Long filiform tail extends posteriorly 0.382 to 0.399 mm. from postcloacal papillae. In one specimen two small cuticular spines present on tail, 0.251 and 0.317 mm. from tail tip. Spicule not visible.

**Female:** 6.40 to 11.52 mm. long; maximum width 0.480 to 1.104 mm.; width at level of vulva varies from 0.384 to 0.792 mm. Lateral cuticular ridges present. Esophagus 0.792 to 0.912 mm. long; bulb 0.099 to 0.138 mm. long and 0.112 to 0.122 mm. wide. Nerve ring 0.268 to 0.313 mm. from anterior extremity. Excretory pore and vulva situated, respectively, 0.816 to 0.980 mm. and 0.960 to 1.200 mm. from anterior end. Terminal portion of reproductive tract thin-walled, preceded by thick-walled muscular ovejector. Ovarian and uterine coils postbulbar. Anus 1.440 to 1.920 mm. from posterior extremity. Filamentous portion of tail bears 10 or 11 cuticular spines. Eggs ellipsoidal and slightly flattened on one side; cuticular knob present at one pole. Eggs undeveloped at deposition, 0.112 to 0.125 mm. long and 0.033 mm. wide.

**Host:** *Sceloporus graciosus vandenburgianus* Cope

**Location:** Large intestine

**Locality:** San Bernardino County, California

**Type:** U. S. Natl. Museum Helminth. Coll. No. 48703.

In having a filiform spiny tail, the female of *P. giganticus* resembles five other species of *Pharyngodon*. Of the five species *P. giganticus* most closely resembles *P. extenuatus* (Rudolphi, 1819) and *P. oxkutzcabensis* Chitwood, 1938. The present species differs from *P. extenuatus* in lacking a spicule and in the size and shape of the eggs. It differs from *P. oxkutzcabensis* in being a much larger form and in having a relatively longer esophagus and fewer spines on the tail of the female. The female of *P. giganticus* attains a greater size than that reported for any other species of *Pharyngodon*.

*Pharyngodon cnemidophori*

(Figs. 5-9)

**Diagnosis: Male:** 2.00 to 2.26 mm. in length, maximum width 0.240 to 0.264 mm. Lateral alae about 0.008 mm. wide, extending posteriorly from point 0.132 to 0.165 mm. behind head; posterior limit of alae 0.264 to 0.432 mm. from posterior end. Esophagus begins 0.008 to 0.016 mm. from anterior extremity, 0.320 to 0.366 mm. in length; esophageal bulb 0.066 to 0.070 mm. long, 0.069 to 0.082 mm. wide. Nerve ring 0.049 to 0.089 mm. from anterior extremity. Excretory pore opens on ventral surface, 0.455 to 0.537 mm. from head. Well developed caudal alae

present, 0.033 to 0.037 mm. wide, 0.089 to 0.099 mm. long, extending to point 0.050 to 0.066 mm. from posterior extremity. Usual three pairs of caudal papillae present, all within caudal alae. The anterior pair slightly anterior to level of cloacal opening and bent medially to support cupped anterior portion of caudal alae. Two posterior pairs of caudal papillae postcloacal. Prominent genital cone present, 0.023 to 0.033 mm. in length. Cloacal opening 0.125 to 0.142 mm. from posterior extremity. Slender tail with bulbous enlargement at level of posterior limit of caudal alae. Spicule, if present, very slightly cuticularized; measurements not feasible.

*Female*: 5.95 to 6.24 mm. long; maximum width 0.600 to 0.672 mm. Esophagus 0.537 to 0.840 mm. long; esophageal bulb 0.096 to 0.109 mm. long, 0.105 to 0.125 mm. wide. Nerve ring 0.148 to 0.198 mm. from anterior extremity. Excretory pore and vulva 1.224 to 1.320 mm. and 1.368 to 1.488 mm., respectively, from anterior end. Ovarian and uterine coils posterior to level of excretory pore. Eggs barrel-shaped, 0.132 to 0.148 mm. long, 0.040 to 0.050 mm. wide. Tail tapers rather abruptly to point. Anus 0.481 to 0.744 mm. from posterior end.

*Host*: *Cnemidophorus tessellatus tessellatus* (Say)

*Location*: Large intestine

*Locality*: San Bernardino County, California

*Type*: U. S. Natl. Museum Helminth. Coll. No. 48705.

*P. cnemidophori* resembles *P. warneri* Harwood described from *Cnemidophorus sexlineatus* in South Texas. The present species differs from *P. warneri* in being a larger form, in having a shorter tail in the female, and in having larger eggs. *P. cnemidophori* differs from *P. papillocauda* Hannum, described from *Cnemidophorus gularae* in Arizona, in that the female of *P. papillocauda* has numerous irregularly distributed papillae on the tail.

*Pharyngodon californiensis* n. sp.

(Figs. 10, 11)

*Diagnosis*: *Male*: 2.59 mm. long, maximum width 0.188 mm. Lateral alae extending posteriorly to anterior limit of caudal alae, about 0.026 mm. wide in this region. Esophagus 0.456 mm. long (including bulb). Bulb 0.049 mm. long, 0.059 mm. wide. Nerve ring and excretory pore 0.039 and 0.600 mm., respectively, from anterior end. Inconspicuous caudal alae present, about 0.015 mm. wide and 0.050 mm. long. Three pairs of caudal papillae present. Precloacal pair on swollen portion of caudal end. Adcloacal pair postero-laterally directed, supporting caudal alae. Mammillate postcloacal papillae lateral in position. Caudal alae extend to level of postcloacal papillae. Prominent cloacal protuberance present. Cloacal opening 0.366 mm. from posterior extremity. Long filiform tail extends posteriorly 0.310 from postanal papillae. Spicule not visible.

*Female*: 5.57 to 7.39 mm. long, maximum width 0.231 to 0.297 mm. Width at level of vulva varies from 0.204 to 0.297 mm. Lateral alae present, extending from level of 0.033 to 0.100 mm. behind head, posteriorly to base of filiform portion of the tail. Esophagus 0.488 to 0.720 mm. long (including bulb). Bulb 0.082 to 0.105 mm. long, 0.089 to 0.105 mm. wide. Nerve ring 0.040 to 0.132 mm. from anterior extremity. Excretory pore and vulva 0.455 to 0.682 mm. and 0.511 to 0.744 mm., respectively, from anterior extremity. Ovarian and uterine coils postbulbar, extending posteriorly to level of anus. Anus 1.176 to 1.440 mm. from posterior extremity. Tail extended as filiform process, 0.912 to 1.080 mm. in length, bearing 9 to 12 posteriorly-directed cuticular spines. Egg thin-shelled, ellipsoidal, slightly flattened on one side; shell markedly thickened at one pole. Eggs embryonated in preserved specimens, 0.125 to 0.138 mm. long by 0.033 to 0.043 mm. wide.

*Host*: *Coleonyx variegatus* (Baird)

*Location*: Large intestine

*Locality*: San Bernardino County, California

*Type*: U. S. Natl. Museum Helminth. Coll. No. 48704.

*P. californiensis* most closely resembles *P. oxkutzcabiensis* Chitwood, described from *Thecadactylus rapicaudus* in Yucatan. The present species differs from *P. oxkutzcabiensis* in being a somewhat larger worm, in having a relatively longer tail, and fewer tail spines in the female. The eggs of the two species differ in shape.

Hannum (1942) reported *P. extenuata* (Rud.) from *Coleonyx* sp. in Arizona. Study of Hannum's brief description and figures indicates that he was probably not

dealing with *P. cxtenuata* but rather with *P. californiensis*. *P. extenuata* is, for the present, not considered by the writers to be present in the North American fauna.

*Pharyngodon cubensis* n. sp.

(Figs. 12-15)

*Diagnosis: Male:* 1.76 to 2.18 mm. long, maximum width 0.175 to 0.227 mm. Lateral alae quite narrow, extending posteriorly from level of esophageal bulb to level of cloacal opening. Nerve ring 0.035 mm. from anterior end. Esophagus 0.310 to 0.305 mm. long; bulbar portion 0.077 to 0.088 mm. in diameter. Excretory pore 0.473 to 0.609 mm. from anterior end; posterior border of excretory pore is ctenoid. Caudal alae supported by two anterior pairs of caudal papillae. Most anterior pair of caudal papillae forked. Posteriormost pair of caudal papillae not included in caudal alae. Prominent cloacal protuberance present. Cloacal opening 0.185 to 0.192 mm. from posterior end. Filiform portion of tail extends posteriorly 0.122 to 0.140 mm. from postcloacal papillae. Spicule not visible.

*Female:* 3.69 to 4.54 mm. long, maximum width 0.470 to 0.487 mm. Head bears three bilobed lips. Inconspicuous lateral cuticular ridges present. Esophagus 0.350 to 0.364 mm. long; bulbar portion 0.105 mm. long by 0.105 to 0.129 mm. wide. Nerve ring 0.080 to 0.090 mm. from anterior extremity. Excretory pore and vulva 0.262 to 0.360 mm. and 0.292 to 0.430 mm., respectively, from anterior end. Anus 0.770 to 0.945 mm. from posterior end. Smooth filiform portion of tail 0.595 to 0.665 mm. long. Eggs pyriform, slightly flattened on one side; knoblike structures present at poles. Eggs 0.158 to 0.162 mm. long, 0.042 to 0.045 mm. wide.

*Host:* *Tarentola americana* Gray

*Location:* Large intestine

*Locality:* Santiago, Cuba.

*Type:* U. S. Natl. Museum Helminth. Coll. No. 48706.

This species is closely related to *P. auziensis* Seurat which occurs in *Tarentola mauritanica* in Spain and North Africa. *P. cubensis* differs from *P. auziensis* in having a longer tail, larger eggs and a shorter esophagus in the female and in the absence of a spicule in the male.

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PLATE I

*Pharyngodon giganticus* n. sp.

- FIG. 1. Egg. FIG. 3. Female, vulvar region, lateral view.
- FIG. 2. Male, cauda, ventral view. FIG. 4. Female cauda, lateral view.

*Pharyngodon cnemidophori* n. sp.

- FIG. 5. Female, anterior end, lateral view. FIG. 7. Male, cauda, lateral view.
- FIG. 6. Female, cauda, lateral view. FIG. 8. Male, cauda, ventral view.
- FIG. 9. Egg.

*Pharyngodon californiensis* n. sp.

- FIG. 10. Male, cauda, ventral view. FIG. 11. Egg.

*Pharyngodon cubensis* n. sp.

- FIG. 12. Male, cauda, lateral view. FIG. 14. Female, cauda, lateral view.
- FIG. 13. Male, cauda, ventral view. FIG. 15. Egg.

PLATE I



THE AXENIC CULTIVATION OF *RHABDITIS BRIGGSÆ* DOUGHERTY AND NIGON, 1949 (NEMATODA: RHABDITIDAE). III.  
LIVER PREPARATIONS WITH VARIOUS  
SUPPLEMENTATION<sup>1</sup>

ELLSWORTH C. DOUGHERTY<sup>2</sup>

Department of Zoology, University of California  
Berkeley, California

The axenic cultivation (*i.e.*, growth in the absence of other living organisms) of the free-living soil nematode, *Rhabditis briggsæ*, requires a complex medium including one or more heat-labile, protein-like substances which have been termed "factor Rb" (Dougherty, 1950, 1951a). It has been shown that factor Rb can be provided by preparations from liver or chick embryo juice (Dougherty, 1951a) or by human plasma or certain of its fractions (Dougherty, 1951b). Moreover, it has recently been reported in abstract (Dougherty and Keith, 1951) that a dialysed fraction of buffered aqueous liver extract will support excellent growth of *R. briggsæ* when supplemented with known substances only.

The present paper reports the results of recent studies on the nature and properties of factor Rb in liver protein and on various supplementations of certain unheated liver preparations as media for *R. briggsæ*. Aqueous, unheated horse liver extract (hereinafter referred to as LE), prepared by centrifuging liver homogenate and taking the supernatant, has been treated in various ways to provide information on the properties of factor Rb. Such preparations have been variously supplemented and tested as media for the axenic cultivation of *R. briggsæ*. The principal supplementation used has been the supernatant (ALE) from autoclaved LE. Both partly and completely defined supplementations have also been employed. From these studies some advance has been made in the direction of a completely defined medium for *R. briggsæ*.

MATERIALS AND METHODS

*General Considerations*

The general techniques of growing *R. briggsæ* in axenic media and preparing larvae from two-membered cultures for experimental use have already been described (Dougherty, Raphael, and Alton, 1950; Dougherty, 1951a). The experiments recorded here were carried on at room temperature except that, when, as occasionally happened, this rose above 21° C, the tubes were placed in a constant temperature room at 18° C.

A number of methods of preparing ALE have been used. The principal one consisted of autoclaving the supernatant from centrifuged liver homogenate (made on a Waring Blendor by mixing equal parts, by weight, of liver and distilled water,

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or of liver and 1/15 M potassium phosphosphate buffer at pH 7) at 15 lbs./in.<sup>2</sup> (= 1 kg./cm.<sup>2</sup>) pressure, mixing the resulting supernatant and precipitate in a Waring Blendor, centrifuging this mixture, and sterilizing the clear supernatant by Seitz-filtration. Centrifugation was done on a Sorvall high-speed centrifuge at maximum speed (about 20,000 G).

Two samples of ALE, both prepared with buffer as described, were found to contain 37 mg. of non-volatile solid per ml. including the buffer, which was originally 10.4 mg. of mixed phosphates per ml.; since buffer and liver had been mixed in equal quantity but a considerable part of the latter spun down to leave the supernatant LE, the former had been subjected to something less than dilution by half—let us assume roughly to 7 mg./ml. Of the remaining 30 mg. about three-quarters consisted of dialysable molecules, and the balance of non-dialysable substances, of which about 3 mg. precipitated down in the presence of 5% trichloroacetic acid and were thus principally protein. Various batches of ALE have probably been somewhat variable of constitution as a result of differences in the details of their preparation.

#### *Chemical Treatment of LE*

LE was treated with 0.1 N acid, 0.1 N base, 8 M urea, and pepsin.

In preparing LE for acid or base treatment distilled water was used. The LE was Seitz-filtered, and the sterile product was found to contain approximately 50 mg. of protein/ml. by dry weight as determined by trichloroacetic acid precipitation. (LE prepared with 1/15 M phosphate buffer is much more difficult to handle than that prepared with distilled water. Even after centrifugation for 15 minutes at over 100,000 G on the Spinco ultracentrifuge the supernatant of the former is not clear and Seitz-filters with great difficulty; furthermore, the filtered product is often not sterile, even when put simultaneously through two pads.)

Sterile LE prepared as indicated was put in sterile bags made of Visking tubing, each having previously been set up as follows. A small test-tube with the blind end cut off was shoved through a tightly fitting hole bored in the rubber stopper of a 2 l. Erlenmeyer flask; this was done in such a manner that only about an inch of the tube projected above the outer side of the stopper. The Visking bag was tied with strong thread to the inner end of the tube, the outer end plugged with cotton, and the system of stopper, tube, and bag sterilized by autoclaving. If, for convenience, the 2.1 Erlenmeyer is also included, the stopper must fit loosely so as to permit gas to go in and out during and after autoclaving; otherwise the Visking bag will rupture. 30 milliliters of LE were added to each of two bags so prepared, and the outer ends of their tubes tightly closed with sterile rubber stoppers.

Two samples of LE were then dialysed on a shaker at 4° C. for twenty-four hours against about 1800 ml. of 0.1 N HCl and of 0.1 N NaOH, respectively. Next the samples were separately dialysed against three changes of distilled water over a thirty-six hour period. The contents of the Visking bags remained sterile and could be removed with sterile pipettes. The LE treated with acid and base in the way described remained clear and formed no precipitate at the end of dialysis. There was in both cases a certain dilution of the non-dialysable material with water entering by reason of osmotic forces; this was not exactly measured, but resulted in but a small increase in volume.

The urea-treated LE was prepared by dialysing approximately 100 ml. of non-

sterile LE (prepared with phosphate buffer) against 1800 ml. of 8 M urea solution (at pH 7) for twenty-four hours according to the same technique as used for acid and alkali treatment. The LE was then dialysed against several changes of distilled water. A copious precipitate formed which was removed by centrifugation, and the clear supernatant was then sterilized by passing it through a Seitz-filter. After this last procedure a considerable amount of flocculation developed in the filtrate on standing. The original LE had approximately 100 mg. of protein/ml. on a dry weight basis; the final product was not measured, but was obviously much less than this.

Peptic digest of LE was prepared by adding 50 mg. of crystalline pepsin to 50 ml. of unsterilized LE (about 5 gms. of protein). The pH was adjusted to 2 with concentrated HCl, and the mixture incubated at 37° for twenty-four hours. During the incubation the pH was readjusted to 2 on two occasions. Untreated sterile LE was incubated as a control. The enzyme-treated mixture was then neutralized with concentrated NaOH and Seitz-filtered.

#### *Fractionation of LE*

Five pounds of horse liver was used to prepare LE. This was made as described, with buffer, but not Seitz-filtered. It was then fractionated with ammonium sulfate as follows, all procedures being carried out at 4° C. A saturated solution of  $(\text{NH}_4)_2\text{SO}_4$ , also prepared at 4° C. and adjusted to pH 7 with concentrated  $\text{NH}_4\text{OH}$ , was added dropwise to the LE with constant stirring. That part precipitating out up to 20% saturation with  $(\text{NH}_4)_2\text{SO}_4$  was centrifuged down and discarded. The supernatant was then increased to 40%  $(\text{NH}_4)_2\text{SO}_4$ , and the precipitate centrifuged down and saved. Similarly the 40–60% and 60–80% fractions were prepared. The 80–100% fraction was prepared by adding crystalline  $(\text{NH}_4)_2\text{SO}_4$  to the supernatant at 80% saturation. The 20–40% fraction was designated LPF-B; the 40–60%, LPF-C; the 60–80%, LPF-D; and the 80–100%, LPF-E. The precipitated fractions LPF-B, -C, and -D were then redissolved in mixtures of buffer and saturated ammonium sulfate solution to give approximately 20, 40, and 60% saturation, respectively, and the precipitations repeated; these procedures were then carried out a third time. Next the three fractions plus the once-precipitated LPF-E were each dissolved in 100 ml. buffer and dialysed against several changes of buffer over a 24–48 hour period. Finally, the portions non-diffusible in dialysis were sterilized by Seitz-filtration.

LPF-B, -C, and -D were tested for protein content by precipitation at 5% trichloroacetic acid and gave values of 21, 37, and 19 mg./ml., respectively; LPF-E was not tested.

A batch of LPF-B was also prepared from liver using distilled water in place of buffer. By spectrophotometric determination this had approximately 10 mg./ml. protein (on the assumption that it was of globulin nature).

#### RESULTS

##### *With Chemically Treated LE*

When equal parts of the preparations of LE, produced by acid, base, urea, and pepsin treatments respectively, were added to buffer-prepared ALE (tubes set up in duplicate) and streptomycin-sterilized larvae of *R. briggsae* introduced into the

media, growth and reproduction of the nematodes ensued in all cases except in that of the peptic digest. In the case of the acid- and base-treated LE a copious precipitate was formed as soon as either clear preparation was added to the ALE; that of the acid-treated LE was finer than that of the base-treated. As with untreated LE this extensive flocculation of the media much impeded inspection. However, considerable reproduction was apparent two weeks after establishment of the cultures.

The results of growth on the acid- and base-treated LE were rather similar. Large vigorous adults developed from the initial larvae, and numerous II generation larvae were produced, but few of these appeared to mature. After forty-four days, when the first series of cultures were discarded, there was little, if any progress past the II generation. By contrast, the urea-treated LE in the same period developed massive cultures in which most of the flocculated protein had disappeared and accumulation of unhatched eggs in large masses was also evident. A second series of tubes testing the acid- and base-treated LE agreed with the earlier. Sterility tests, as in previous studies (Dougherty, Raphael, and Alton, 1950; Dougherty, 1951a) were carried out on all cultures.

In the case of the peptic digest the medium remained clear, and it could be seen that the larvae did not grow. The tiniest ones curled up and were almost inert, but survived for several weeks. On the supposition that peptic digestion released peptides and amino acids from LE at concentrations toxic for *R. briggsae*, dilutions of the digest were tested. In dilutions from 1/2 to 1/8 of that in the original series the larvae introduced remained active; but no maturation of small larvae was evident at concentrations down to 1/8 of the original. The control LE, which had been incubated at 37° C. along with the LE being peptically digested, provided for development of large adults, but no reproduction was evident.

The foregoing series were controlled with tubes containing untreated LE supplemented with ALE; these tubes, unlike those of previous experiments with calf LE, supported only the maturation and not the reproduction of the inoculated larvae.

The results of the experiments reported up to this point are summarized in table 1.

TABLE 1.—Growth of *R. briggsae* on horse LE variously treated and supplemented with ALE

Sources of factor Rb	Untreated LE	LE incubated at 37° C.	0.1 N HCl-treated LE	0.1 N NaOH-treated LE	8 M urea-treated LE	Peptic digest of LE
Autoclaved protein (mg./ml. by dry wt.) in final medium	25	25	< 25	< 25	≤ 50	Negligible
Type of growth <sup>1</sup>	+	+	++	++	+++	—

<sup>1</sup> — = no maturation; + = maturation without reproduction; ++ = maturation with considerable reproduction, but without establishment of thriving culture; +++ = establishment of thriving culture.

#### With LE Protein Fractions (LPF)

When freshly prepared LPFs, supplemented with equal parts of buffer-prepared ALE, were tested, maturation and reproduction of *R. briggsae* occurred with LPF-B, -C, and -D, but not with -E. Of these LPF-C was somewhat more active than LPF-B, and both -B and -C were far more active than -D. With LPF-C and -B vigorous cultures consisting of three or more generations were uniformly produced, whereas with -D there was little if any advance beyond the production of II-generation larvae.

Quite fortuitously LPF-B was retested after remaining in the ice box at 4° C. for a 4-month period and, surprisingly enough, proved much more active than the freshly prepared material. Its sterility was beyond question. It was markedly superior even to fresh LPF-C, which to date has not been tested after aging. This superiority was best evident when results of using aged LPF-B (21 mg./ml.) were compared with those using a comparable dilution (half-strength) of LPF-C (full strength = 37 mg./ml.), for, although growth was no faster in the former (maturation in about eight days), by about two weeks the forms therein could be seen to be more numerous, larger, and more vigorous than in LPF-C. By contrast LPF-B incubated at 37° C. for 4 days showed considerable precipitation and proved almost inactive.

LPF-C was tested at full strength and at dilutions (with buffer) of 1/2, 1/4, and 1/8—in each case further diluted in half with ALE in the final medium. Good cultures were produced at dilutions down to 1/4 (9 mg./ml.—further diluted to half by equal parts of ALE), but growth was progressively slower, the culture reaching its acme at a progressively later date. At a dilution of 1/8 there was little if any advance beyond the I generation.

Supplementation of LPF-C and -B with only known ingredients provided very interesting results. Four groups of known substances were compounded, based on portions of the Medium II used previously with *R. briggsae* (Dougherty, Raphael, and Alton, 1950) as a supplement of chick embryo juice. These were dissolved in buffer and were: 1) vitamins and growth factors (excluding cholesterol, and with thiamine at 2.0  $\gamma$ /ml. (as opposed to 3.5) and choline at 20 (as opposed to 22)); 2) energy sources (*i.e.*, glucose and sodium acetate); 3) nucleotides (plus thymine); and 4) salts (excluding phosphate, which was already present in the buffer), and KCl).

It was found with both LPF-C and aged LPF-B that growth and reproduction occurred when the supplementation included groups 1, 2, and 4; in the case of LPF-C growth was inferior to that with ALE supplementation, but with aged LPF-B, growth was very vigorous and equal to that with ALE. By contrast freshly prepared LPF-B was almost inactive when thus supplemented. This known supplementation may be designated VES (vitamins-energy sources-salts).

With LPF-C tests were made with supplementation by all groups (1–4) of known ingredients and with the single omission of groups 1, 2, 3, or 4 from the mixture. It was found that the omission of vitamins, energy sources, or salts retarded or prevented maturation and completely prevented reproduction. On the other hand the presence or absence of nucleotides (plus thymine) made no apparent difference to the end result—a vigorous culture resulted.

LPF-B and -C were submitted to certain analyses. With the Beckman Spectrophotometer they give 280/260  $m\mu$  absorption coefficients that indicated a maximum nucleic acid content of not more than 0.25%, as calculated from data given by Warburg and Christian (1941, p. 402). With the Spinco Analytical Ultracentrifuge LPF-C was shown to consist of three detectable components with different rates of sedimentation. Analysis for fats by Dr. Harold T. Gordon showed that it had less than 20  $\gamma$  of total lipids per ml. (unpublished micro-method using decolorization of ceric sulfate).

The addition of groups 1, 2, 3, and 4 individually to the LPF-C plus ALE medium, in which ALE was 4/5 as concentrated as in the unsupplemented combination (except for the combination of energy sources and ALE, in which the latter was at full strength), but in which the known ingredients were present in the same concentration as in the experiments already described, resulted in no obvious improvement in growth or reproduction.

The inclusion of certain additional substances in the LPF-C plus VES medium was also tried. These substances included (with final concentration in the medium): 1) cyanocobalamine (or vitamin B<sub>12</sub>—0.001  $\gamma$ /ml.); and 2) a combination of equal parts of linseed and cod-liver oil (250  $\gamma$ /ml., partly stabilized with Tween 85—ca. 25–50  $\gamma$ /ml.), designed to provide unsaturated fatty acids; in addition choline was increased from 20 to 400  $\gamma$ /ml. With these three supplementations added singly and in all possible combinations no improvement in growth or reproduction was noted. In fact the presence of oil plus Tween was decidedly inhibitory and few adults were produced, with little or no reproduction.

LPF-B prepared with distilled water instead of 1/15 M phosphate buffer proved to be inactive when supplemented with VES likewise prepared with water (with the addition of 100  $\gamma$ /ml. of K<sub>2</sub>HPO<sub>4</sub> and of KH<sub>2</sub>PO<sub>4</sub>, respectively, to the S mixture); but, when the former was dialysed against buffer and then retested against the same VES, it proved active. In this case the aging period had been two months. Water-prepared LPF-B, supplemented with water-prepared ALE, also proved active. When unprotected by buffer or ALE, considerable LPF-B could be seen to precipitate out of solution in a few days of standing. Finally, the water-prepared LPF-B, dialysed against buffer, was supplemented with that part of ALE diffusible in dialysis; this combination also proved active.

The results of experiments with LPFs are summarized in table 2.

TABLE 2.—Growth of *R. briggsae* on LPF with various supplementations<sup>1</sup>

LPF <sup>2</sup>	Range in which precipitated by (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Amount in final medium (mg./ml.)	Supplementation <sup>2</sup>	Results <sup>3</sup>
LPF-B (fresh)	20–40%	10.5	ALE VES	+++ ±
(aged at 4° C.)		10.5	ALE <sup>4</sup> VES	++++ ++++
(incubated at 37° C.)		?	ALE VES	± —
LPF-B [water]	20–40%	5.0	ALE [water] VES [water]	+++ —
LPF-C (fresh) full strength	40–60%	18.5	ALE <sup>5</sup> VES <sup>6</sup>	+++ +++
1/2 “	“	9.3	ALE	+++
1/4 “	“	4.6	ALE	++(+)
1/8 “	“	2.3	ALE	+
LPF-D (fresh)	60–80%	8.5	ALE	++
LPF-E (fresh)	80–100%	?	ALE	—

<sup>1</sup> LPF = liver protein fraction(s) prepared from aqueous liver extract (LE); ALE = supernatant from autoclaved LE; VES = vitamins, energy sources, mineral salts (as given for Medium II by Dougherty, Raphael, and Alton (1950)—with modifications noted in text of present paper).

<sup>2</sup> Except where “water” is placed in brackets, LPF and their dilutions and supplementations were prepared with 1/15 M K phosphate buffer at pH 7.

<sup>3</sup> Results indicated as in Table 1 except that +++ = thriving culture qualitatively superior to ++.

<sup>4</sup> The dialysable part of ALE also active when supplementing aged LPF-B.

<sup>5</sup> Additional supplementations with V, E, or S components or with nucleotides (plus thymine) did not improve growth.

<sup>6</sup> Additional supplementations with cyanocobalamine (vitamin B<sub>12</sub>), greatly increased choline (from 20 up to 400  $\gamma$ /ml.), linseed + cod liver oil, or nucleotides (plus thymine) brought about no improvement in growth.

## DISCUSSION

*Chemical Effects on LE*

It is obvious that 0.1 N acid and 0.1 N base, acting for several hours at 4° C., at most only partly destroy factor Rb in LE; and 8M urea appears not to destroy it at all. The fact that the untreated horse LE used to control these experiments failed to support reproduction of worms maturing in a medium containing it plus ALE, in contrast with results of previous experiments using calf LE (Dougherty, 1951a), cannot be completely explained without additional investigation. It should be pointed out that in reporting these studies on calf LE, I described its preparation as being carried out with distilled water; actually tests on LE supplemented with ALE had been done with buffer-prepared LE and ALE, whereas most of the studies on LE supplemented with known ingredients had been done with LE prepared with distilled water. Since the work with LPF-B reported here has established that this fraction has greater activity when dissolved in buffer than when dissolved in distilled water, it seems not unlikely that the horse LE used in the studies under consideration was relatively inactive by reason of lack of buffer; factor Rb activity may have been blocked by the precipitating out of much of the protein containing it, which was then indigestible to the worms.

It is interesting in light of the foregoing facts that the worms actually did better on the acid and alkali-treated LE and that on urea-treated LE they did very well indeed. Toxic factors in the whole LE may have been eliminated by dialysis. It is also possible that the natural protein containing factor Rb is broken down by such treatments, particularly by urea, into simpler protein moieties that are dietetically more readily available to the worms.

At the present time it has not been possible to produce a non-protein source of factor Rb. However, it can be stated with reasonable likelihood that factor Rb need not be present as a native protein. Urea at high concentrations is an extremely powerful denaturing agent (Neurath *et al.*, 1944); and it seems unlikely that a native protein could withstand, unaltered, the several actions of urea and of relatively strong acid and base. One is, therefore, led to relegate to improbability any hypothesis that factor Rb is an enzyme; however, the possibility cannot be completely dismissed.

If factor Rb is a protein substituent of non-protein or of peptide nature, it is not released in a form readily diffusible in dialysis by the action of 8 M urea or of 0.1 N acid or base. Further work is necessary to determine if factor Rb can be salted out with ammonium sulfate after any of these three treatments.

Destruction of factor Rb by peptic digestion may be due to the splitting of factor Rb itself, or to its destruction, when freed, at the acid pH of the process. Further efforts at protein hydrolysis, using other proteolytic enzymes to release a smaller non-protein molecule with factor Rb activity, are appropriate.

*Use of Liver Protein Fractions*

The growth of *R. briggsae* on dialysed LPF-B or -C plus defined (VES) supplementation only, represents a considerable step forward toward a completely defined medium for this species. When this was partly reported in abstract recently (Dougherty and Keith, 1951) mention was made only of the use of LPF-B because

of its apparent superiority over LPF-C; but at that time the aging effect had not been recognized. It is interesting to note that the purine and pyrimidine needs of *Paramecium multimicronucleatum* can be supplied in a heat-labile, protein-like form that is very similar to factor Rb in its properties, including a somewhat analogous aging effect (see Johnson and Tatum, 1945; Johnson, 1952). When heated to 55° C. for 30 min., it has been shown to lose its activity, but this is regained in considerable part when the preparation is allowed to age at 10° C. for two weeks.

It seems likely that aging, which has been tested on LPF-B, but not yet on -C, results in the autolysis of the former, releasing smaller, digestible molecules. The effect of urea might be similar. These molecules would not appear to be very small, however, inasmuch as dialysis of two month-old, water-prepared LPF-B against buffer did not result in loss of activity by diffusion through the dialysis membrane. The possibility that one or more such molecular species may not be factor Rb is discussed in the following paper (Dougherty and Keith, 1953). In any event, in light of the aging effect, factor Rb may be present in fresh liver protein partly in a bound form or forms that are not available to *R. briggsae*.

It may well be that the heat-lability of factor Rb is due to nothing more than the rendering digestively unavailable, by coagulation, of those proteins that contain as a structural substituent an otherwise heat-stable, essential molecule. This interpretation is given strength by the recent observation of Johnson (1952) on the already mentioned, heat-labile pressed yeast juice factor previously thought to be required by *P. multimicronucleatum*, namely, that it can be replaced with heat-stable purines and pyrimidines (which do not, however, replace factor Rb for *R. briggsae*); here a digestibility problem seems clearly involved.

Comparison of the LPF plus VES media reported on here with the completely defined media that support excellent growth of ciliates of the genus *Tetrahymena* (see Kidder and Dewey, 1951, p. 392)—the only true animals that to date can be grown indefinitely and well on such media—reveals significant differences. With more or less comparable concentrations of known ingredients in the two groups of media—other than free amino acids, which are lacking as such in the *R. briggsae* media, *R. briggsae* appears to require for optimal growth a much higher absolute amount of amino acids (as protein) than does *Tetrahymena* (as free acids). *Tetrahymena* does exceedingly well, for example, on a protein-free medium (A) containing a total of 3.37 mg. of amino acids per ml. *R. briggsae*, however, has been shown to require at least 4 mg. of fresh LPF-C per ml. plus the protein of ALE (1.5 mg./ml.) for the production of vigorous cultures, and 9 and 18 mg. have shown increasingly marked, stimulating effects. As already noted, when supplemented with VES, 18 mg./ml. of fresh LPF-C gave slower growth and less vigorous cultures than when supplemented with ALE. On the other hand 10 mg./ml. of aged LPF-B gave very vigorous cultures when supplemented either with ALE or VES.

The foregoing data again suggest a digestibility problem. So far free amino acids have not been specifically tested for their ability to spare intact protein in *R. briggsae* nutrition. However, even when ALE, which contains proteins and, in view of such data as those of Awapara, Landua, and Fuerst (1950), peptides and free amino acids as well, is used as a supplementation for LPF-C, the absolute requirement for the latter is still relatively very high. It seems more than likely, therefore, that the amount of factor Rb available establishes the lower limit of

LPF required in the medium. It is not possible to say, however, whether this requirement is for a micronutrient or for a substance or substances needed in moderate or even large amounts.

The preparation of LPF-B with distilled water instead of buffer was carried out on the supposition that the high concentration of potassium (3.9 mg./ml.) might be toxic to the worms. The failure of growth in the presence of VES without high levels of phosphate buffer appears definitely related to the effect of the latter in stabilizing LPF-B in solution. The protein and salts of water-prepared ALE have a similar protective action.

The fact that factor Rb is mostly confined to the protein fractions coming down in the range 20–60% saturation with  $(\text{NH}_4)_2\text{SO}_4$ , being scanty in the 60–80% and absent in the 80–100% fraction, demonstrates that it is apparently associated with the globulins in liver protein and suggests that it may itself be a globular protein.

Of the VES components none has as yet been demonstrated to be an absolute requirement for *R. briggsae*. However, from the results of experiments in which V, E, and S were individually left out as groups, it is evident that *R. briggsae* requires for adequate growth one or more water soluble vitamins, a carbohydrate energy source, and certain mineral salts. The need for pre-formed purines and pyrimidines cannot be established or excluded so long as they have not been definitely eliminated from LPF or other components of the medium. Fat soluble substances do not seem to be required inasmuch as LPF-C has negligible fat, and VES supplies none. Finally, amino acid requirements may reasonably be assumed, but even in the best defined media these have been so far satisfied by the LPF.

Table 3 summarizes the major constituents of the best axenic liver medium so far devised for *R. briggsae*.

TABLE 3.—Major components of aged LPF-B + VES medium for *R. briggsae*<sup>1</sup>

	Absolute amount (mg./ml.)	Percentage (by dry wt.)
Energy sources	2.00	8.6%
Liver protein	10.50	45.4%
Mineral salts	10.52 <sup>2</sup>	45.4%
Nucleotides or their bases	(0.026) <sup>3</sup>	(0.11%)
Vitamins	0.102	0.44%

<sup>1</sup> For abbreviations see table 2.

<sup>2</sup> The high value is due to the use of 1/15 M K phosphate buffer at pH 7 (10.4 mg./ml.).

<sup>3</sup> This is the maximum amount possible in LPF-B by spectrophotometric measurement; the actual amount may be much less than this.

#### SUMMARY

1. Factor Rb in unheated aqueous liver extract (LE) has been shown to resist urea denaturation and, at least partly, the action of 0.1 N acid and 0.1 N base under conditions tested. Activity for *R. briggsae* is retained in the treated portion of LE non-diffusible through a dialysis membrane.

2. Peptic digestion has failed to release a smaller, non-protein molecule with factor Rb activity. However, it appears somewhat unlikely that factor Rb has to be in the form of a native protein in order to support growth of *R. briggsae*; it is thus unlikely that it is an enzyme.

3. LE protein has been fractionated with  $(\text{NH}_4)_2\text{SO}_4$  into parts coming down at 20–40% (LPF-B), 40–60% (-C), 60–80% (-D), and 80–100% (-E) saturation at 4° C. Of these, in the fresh state, LPF-C is most active as a source of fac-

tor Rb. On aging at 4° C for 2 or more months LPF-B becomes more active than fresh LPF-C; aged LPF-C has not been tested. Factor Rb is apparently associated with the globulin components of liver protein.

4. Fresh LPF-B and LPF-C, prepared with buffer, support good cultures when supplemented with the supernatant (ALE) from autoclaved LE. Fresh LPF-C and aged (but not fresh) LPF-B also support good cultures when supplemented with known materials only (VES)—certain vitamins, carbohydrate energy sources, and mineral salts.

5. If V, E, or S are left out when VES would otherwise be the sole supplementation to LPF, little or no growth ensues.

6. If prepared with water instead of buffer and supplemented with water-prepared VES, aged LPF-B does not support growth. It does support growth, however, if supplemented with water-prepared ALE.

7. It appears likely that, in fresh liver protein, part of the factor Rb may be bound in a form unavailable to *R. briggsae*. Aging at 4° C. may release this, at least in part. The absolute need for protein is very high, but the available amount of factor Rb is probably the limiting factor.

8. No specific known substance has as yet been demonstrated as an absolute requirement for *R. briggsae* (except, of course, water). One or more water-soluble vitamins, a carbohydrate energy source, and certain mineral salts appear essential. Various additional supplementations of LPF + ALH and/or VES media have been tested without improving growth.

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THE AXENIC CULTIVATION OF *RHABDITIS BRIGGSÆ*  
DOUGHERTY AND NIGON, 1949 (NEMATODA:  
RHABDITIDÆ). IV. PLASMA PROTEIN  
FRACTIONS WITH VARIOUS  
SUPPLEMENTATION<sup>1, 2</sup>

ELLSWORTH C. DOUGHERTY AND DOUGLAS F. KEITH

Department of Zoology, University of California, Berkeley, California

The immediately preceding paper (Dougherty, 1953) has been confined to an account of work with liver as a source of factor Rb. In the present paper we deal with studies using human plasma as a source of this substance (or substances). A preliminary note briefly reporting a part of the results has already appeared (Dougherty, 1951).

Whole plasma and its protein fractions and certain subfractions (see Haurowitz, 1950, pp. 148-160) have been tested. These studies throw additional light on the nature of factor Rb.

MATERIALS AND METHODS

Ultraviolet-irradiated human plasma and various of its protein fractions (PPF) were obtained in the lyophilized (dry) state. The former contained sodium citrate anticoagulant (0.4%). Distilled water was added to restore the whole plasma to its original volume; and the solution was Seitz-filtered. Part of the product was dialysed using sterile precautions (as described by Dougherty, 1953) against several changes of distilled water at 4° C. Both untreated plasma and that part (principally protein) non-diffusible in dialysis were tested for their capacity to support growth and reproduction of *R. briggsæ*; they were supplemented with equal parts of ALE prepared with distilled water.

Fractions and subfractions of plasma protein, the constitutions of which are given in Table 1, were dissolved in 1/15M K phosphate buffer at pH 7 to give 50 mg./ml. Solution was best accomplished by attaching a flask containing the dry powder to a vacuum line and drawing the buffer into the flask; this was achieved by use of a stopper with two holes and appropriate glass and rubber tubing connected therewith. Water under reduced gas pressure penetrates the fluffy protein fractions better than at atmospheric pressure. The fractions were then Seitz-filtered, and 1 ml. aliquots were dried in a drying oven and weighed to determine the protein content (with an allowance made for 10.4 mg. of mixed potassium phosphates). It should be remarked that in the case of PPF-III considerable lipid material remained on the filter pad. Finally these fractions were tested for their ability to support growth and reproduction of *R. briggsæ*; they were supplemented with buffer-prepared ALE and in certain cases with other preparations.

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<sup>1</sup> These studies were largely done while the senior author was a Research Fellow of the American Cancer Society as recommended by the Committee on Growth of the National Research Council, 1949-52.

<sup>2</sup> We are much indebted to Dr. Karol A. Hok, Cutter Laboratories, Berkeley, for the human plasma and its protein fractions used in the work reported here.

The dialysable and non-dialysable fractions of ALE were prepared for testing as supplements to PPF-III. About 100 ml. of buffer-prepared ALE were dialysed against three changes of a large excess of distilled water and finally against buffer; this was the non-diffusible portion (*nd*). The diffusible portion (*d*) was prepared by dialysing a large excess of ALE against about 30 ml. of sterile buffer contained in a dialysis bag.

Buffer-prepared VES was also tested as a supplement to certain fractions and subfractions.

TABLE 1.—*Growth of R. briggsae on plasma protein fractions supplemented with ALE or VES*<sup>1</sup>

PPF	Constitution (in per cent) <sup>2</sup>						Amount in final medium (mg./ml.)	Results with supplementation <sup>3</sup>	
	Albumin	$\alpha_1, \alpha_2$	$\beta_1$	$\beta_2$	$\phi$	$\gamma$		ALE	VES
I	7	8	15	61	9	14.4	—	—	Not tested
II	0	0	2	(includ- small % of $\phi$ )	98	16.8	+	—	—
III	3	15	33	34 (includ- small % of $\phi$ )	15	17.5	+++	±	—
IV	8	60	31	0	1	16.1	++(+)	—	Not tested
IV-1	0	89	10	0	—	19.3	++	—	—
IV-4	16	46	38	0	0	15.7	++	—	—
IV-1 + IV-4	8	67.5	24	0	0.5	17.5	++(+)	—	—
V	95	4	1	0	0	20.0	—	—	Not tested

<sup>1</sup> ALE = supernatant from autoclaved liver extract; VES = defined supplementation (vitamins, carbohydrate energy sources, mineral salts).

<sup>2</sup> Figures supplied by Dr. Hok;  $\alpha_1, \alpha_2$  =  $\alpha$ -globulins;  $\beta_1, \beta_2$  =  $\beta$ -globulins;  $\phi$  = fibrinogen;  $\gamma$  =  $\gamma$ -globulins.

<sup>3</sup> For estimations of growth see Dougherty (1953, table 1).

A number of more or less heat-labile substances were tested for their ability to replace PPF-III and as supplements to PPF-III + ALE and PPF-III + VES. These included (with their concentrations in the final medium in  $\gamma$ /ml.): 1) co-carboxylase (diphosphothiamine—0.1); 2) coenzyme I (diphosphopyridine nucleotide, or DPN—0.1); 3) coenzyme II (triphosphopyridine nucleotide, or TPN—0.1); 4) pyridoxal phosphate (0.1); 5) riboflavine phosphate (0.1); and 6) glutamine (1.0).

#### RESULTS

Whole plasma, PPF-III, and PPF-IV (and certain of its subfractions), supplemented with ALE, proved to be good sources of factor Rb; PPF-III and -IV (and subfractions) are rich in  $\alpha$ - and/or  $\beta$ -globulins. PPF-II ( $\gamma$ -globulin) was a poor source. And PPF-I (fibrinogen) and -V (albumin) were inactive.

The replacement of ALE with VES as a supplement to PPF-II, -III, and -IV (and certain of its subfractions) resulted in no, or, in the case of PPF-III, very limited, growth. Surprisingly, when ALE was replaced with ALE-*d*, little growth took place; ALE-*nd* was a better supplement, fair cultures resulting; but it took a reconstituted ALE-*d* + *nd* to restore growth to about the level of the original ALE (ALE-*d* + *nd* equaled half-strength ALE).

No one or more of the five co-enzymes tested as a group appeared to be able to replace unheated protein as a factor Rb source, nor could glutamine. When added as a group to ALE by itself, these heat labile factors (HLF) did not provide for the growth of *R. briggsae*, likewise when added to PPF-III + VES. No stimulating effect was evident when HLF were added to PPF-III + ALE.

Results of PPF supplemented with ALE and VES are summarized in table 1.

## DISCUSSION

The foregoing results illustrate that human plasma protein is a good source of factor Rb, which is present in adequate amounts only in the globulin fractions thereof. PPF-III, the most active fraction, is almost 77%  $\beta$ -globulin. The somewhat less active PPF-IV and its subfractions range from 10–38%  $\beta$ -globulin. The  $\gamma$ -globulin fraction (PPF-11), which showed slight activity, has a small amount (slightly less than 2%) of  $\beta$ -globulin and no measurable  $\alpha$ -globulin present. One is tempted therefore to suggest that factor Rb is contained in the  $\beta$ -globulin part of plasma protein. However, PPF-I, which is predominantly fibrinogen (61%), but contains 15%  $\beta$ -globulin, does not support *R. briggsae*.

In any event the limited evidence (Dougherty, 1953) that factor Rb is associated with globulins in liver is much reinforced by the results with the more highly fractionated plasma components.

An interesting difference between the most active plasma protein fraction, PPF-III, and certain liver protein fractions, namely LPF-C and aged LPF-B, resides in the fact that VES is not an adequate supplementation in the former case, whereas it is in the latter. When the further fact is considered that neither is ALE-*d* active as a supplementation to PPF-III, whereas ALE-*nd* by itself restores part of the activity and ALE-*d* + *nd* most of the activity of the combination PPF-III + ALE, it becomes rather suggestive that ALE contains one or more species of large, heat-stable molecules required by *R. briggsae* and occurring in an available form in LPF-C and aged LPF-B, but not in PPF-III (or -IV) or in fresh LPF-B. It is possible, therefore, that in the case of LPF-B the essential thing that the aging process does is to release not previously unavailable factor Rb, but rather a nutritionally important large, heat-stable molecule (or molecules). However, the opposite interpretation is equally tenable.

Factor Rb does not appear to be any one or more of the group of HLF tested—that is, five co-enzymes and glutamine. None of these is heat-labile to the extent of factor Rb. Inasmuch as ALE contains 3 mg./ml. of protein (Dougherty, 1953) there should be sufficient protein nitrogen therein for the amino acid needs of *R. briggsae*—if one assumes that this is digestible protein. Under such circumstances, if factor Rb were a micronutrient requirement (or requirements), it should be possible to obtain good growth by the addition of minute quantities to ALE. This is not true for the HLF as a group.

## SUMMARY

1. Factor Rb is present in human plasma and is apparently confined to the globulin fractions, wherein it most probably is associated with the  $\beta$ -globulins.
2. The most active plasma protein fraction studied, PPF-III, supports good cultures of *R. briggsae* when supplemented with ALE but not with VES.
3. Evidence exists that *R. briggsae* requires one or more species of large, heat-stable molecules, non-diffusible in dialysis, in addition to heat-labile factor Rb. The aging effect of LPF-B may be to release these molecules rather than factor Rb, although the opposite interpretation is equally tenable.
4. Factor Rb does not appear to be glutamine, nor any one or more of five rela-

tively heat-labile co-enzymes (diphosphothiamine, DPN, TPN, pyridoxal phosphate, or riboflavine phosphate).

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# STUDIES ON THE FORMATION AND FUNCTION OF MUCOID GLANDS IN CERCARIAE: OPISTHORCHOID CERCARIAE

FRANCIS J. KRUIDENIER

Contribution from the Departments of Zoology of the University of Michigan and the University of Illinois

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## INTRODUCTION

The demonstration of metachromatic glands in the xiphidiocercariae (Kruidenier, 1951, 1953) and the role of these glands in their economy leads to speculation concerning the presence of similar glands in related cercariae. The discovery of metachromatic substances at the anterior ends of emerged opisthorchoid cercariae led to further studies of available species of these cercariae. Once divided between monostome and distome cercariae (Lühe, 1909, Sewell, 1922) because of extreme variations in their acetabula, these cercariae are currently included in a single group (Faust, 1924, Dubois, 1929, Sewell, 1931). Price (1939, 1940) concluded that cercarial characteristics could not be correlated sufficiently to permit the establishment of natural subdivisions of the cercariae of the superfamily OPISTHORCHIOIDEA.

## MATERIALS AND METHODS

In the present study four species of opisthorchoid cercariae are included: the cercariae of *Euryhelminis monorchis* Ameel, 1938 (Heterophyinae), *Caecincola parvulus* Marshall and Gilbert, 1905 (Cryptogoniminae), and two as yet undescribed species. *Euryhelminis monorchis* was obtained from *Pomatiopsis lapidaria*: *Caecincola parvulus* from *Amnicola* (*Marstonia*) *lustrica*: and the two undescribed species from *Amnicola* (*Amnicola*) *limosa* and *Goniobasis livescens*, respectively. All were collected near Ann Arbor, Michigan.

Whole mounts of normally emerged and of developing cercariae, fixed in basic lead acetate, and serial sections of infected snails, fixed in Bouin's solution were employed in the studies of *Euryhelminis monorchis* and of the undetermined species of cercaria from *Amnicola limosa*. *Caecincola parvulus* was studied from preparations of normally emerged cercariae and from Bouin's fixed, serially sectioned *Amnicola lustrica*. The cercariae from *Goniobasis livescens* were studied from serially sectioned, Bouin's fixed snails. Dilute, aqueous toluidine blue and thionin solutions were used exclusively.

Basic lead acetate is a poor fixative and killing agent. However, usable preparations were obtained with hot, saturated solutions of this reagent, followed shortly with 70% alcohol. Metachromatic differentiation following Bouin's solution was excellent. The concurrent decalcification of the shells when snails were fixed in Bouin's made dissection unnecessary.

## MUCOID GLANDS

In addition to penetration and cystogenous glands, all of the cercariae possess a series of unicellular glands which stain a brilliant metachromatic-red in thionin or toluidine blue. These cells are small in comparison with similar cells described in the xiphidiocercariae (Kruidenier, 1951, 1953) but their metachromasy is as distinct. The secretion of the glands is thus presumably a combination of hexosamine and protein moieties (Lison, 1935) and either a mucopolysaccharide or a mucoid in the classification of Meyer (1945).

The cytoplasm of the mucoid cells appears homogeneous. Nucleoli can occasionally be seen in their large, faintly differentiated nuclei. The glands are paired and lie in a parallel series along the mid-ventral axis of the cercariae. They extend from the oral sucker to the excretory bladder. Dubois (1929) described six, paired, finely granular "ventral cells" with rounded nuclei in *Cercaria lophocerca* (Fil.). It is probable that these cells are identical with certain of the mucoid glands reported here.

The mucoid glands are similar in the species of opisthorchoid cercariae studied. They never penetrate very deeply into the cercarial tissues. All are moderately dendritic (Figs. 2, 4). Their irregular, branching processes are directed ventro-laterally. Four pairs of glands develop between the oral sucker and the acetabulum and one pair forms posterior to the latter organ (Figs. 2, 4, 9).

The anterior pair of glands frequently overlap the oral sucker. Their ducts lead antero-laterad between the ventral surface and the oral sucker, recurve around the sucker and empty through pores located anterior to the dorsal lip of the buccal cavity. Ducts from the second and third pairs of glands lead anteriad and laterad, also encircling the oral sucker. Their pores, near those of the first pair, are located along the edges of a slight pit or depression anterior to the buccal cavity (Figs. 5, 7). The fourth pair of glands lies along the anterior border of the ventral sucker. Ducts from this pair of glands lead around the acetabulum and reproductive fundament to the posterior margins of the body. These ducts are frequently lost from view as they pass closely the pair of glands posterior to the acetabulum.

The glands posterior to the acetabulum possess enlarged ducts. These lead posteriorly, encircle a slight depression at the base of the tail and empty marginally on either side of the body. A small accumulation of mucoid frequently forms in the curved portion of these ducts, simulating an extra pair of glands at the base of the tail.

An interpretation of the functional significance of the mucoid glands requires a knowledge of the levels of cercarial development at which the glands form, mature, and discharge. The progressive development of eye spots in *Caecincola parvulus* and in the cercariae from *Amnicola limosa* and *Goniobasis livescens* and the formation and dispersal of other pigment particles in the last two species afford convenient criteria. Such development parallels that of the other structures of the cercariae. Pigmentation is not affected by the techniques employed, nor does its demonstration depend on technical manipulations. *Euryhelms monorchis* and *Caecincola parvulus* are non-pigmented and eye spots do not develop in *E. monorchis*.

The opisthorchoid cercariae leave their rediae early in their development and

emerge into the adjacent tissues of the snail where they continue their development and growth. The metachromatic differentiation of the mucoid glands depends on the presence of mucoid secretions. Their history prior to the active production of mucoids was not determined. In no instance were the glands visible in cercariae within rediae. Oral sucker, acetabulum, and tail (Fig. 1) are differentiated and the reproductive fundament is visible before the glands appear.

The penetration glands are also distinguishable as the mucoid glands begin to secrete. The bulk of the cytoplasm of the former is non-chromophilic to toluidine blue. However, distinct films of darkly metachromatic substances form against the nuclear and cell membranes of the penetration glands. Their purple-black reaction to toluidine blue indicates that the presumptive mucopolysaccharide or mucoid in the penetration glands is not the same substance as that produced by the mucoid glands. Its sparse distribution indicates that it was produced only in the early history of the penetration glands, before their final differentiation. The non-chromophilic cytoplasm which forms the bulk of their secretions doubtless contains the histolytic substance commonly ascribed to them. Their early production of a mucoid substance may indicate a differentiation of penetration gland cells from that primordium which also gives rise to the mucoid cells.

Eye spots are delimited and strands of pigment are visible in the cercariae from *Amnicola limosa* when their metachromatic glands first appear. Small, irregular masses of mucoid at first closely surround the nuclei of the cells (Figs. 1, 8). Their ducts fill with mucoid almost as soon as the glands can be differentiated. The cells mature rapidly. The initial metachromatic masses indicate the multiple centers of mucoid formation. These masses enlarge and then coalesce. The cells fill completely with mucoid and enlarge appreciably. Concurrently, the pigmentation in the species of cercariae from *Amnicola limosa* (Fig. 4) and *Goniobasis livescens* is distributed into discrete, scattered masses. In these species and in *Caecicola parvulus* the eye spots complete their formation as the mucoid glands attain maximum development.

The cercariae from *Amnicola limosa* and *Goniobasis livescens* discharge their complement of mucoid glands while the pigment of those cercariae is still distributed in numerous, interconnected, but scattered aggregates. In several specimens of nearly mature *Euryhormis monorchis* only the glands posterior to the acetabulum remained. This demonstrates the slightly earlier discharge of the anterior glands. The paucity of such specimens implies the almost simultaneous discharge of all of the glands. The glands of *Caecicola parvulus* also discharge before full maturity is attained in the snail host. It could not be determined how long the opisthorchoid cercariae remained in the snail tissues after the discharge of their mucoid glands. The large number of older, apparently mature cercariae in the snail tissues, outside of but near the rediae of all four species studied, probably indicate that final maturational changes occur before the cercariae emerge from the snail. In addition, several visible changes occur after the discharge of mucoid substances. The pigment particles distribute to form the finely disperse pattern of the normally emerged cercariae (Fig. 6) in the species from *Amnicola limosa* and *Goniobasis livescens*. As in other cercariae (Kruidenier, 1951, 1953), specimens of opisthorchoids are distinctly more chromophilic to thionin and toluidine blue prior to the discharge of their glands. The reproductive fundaments remain dis-

tinctly more chromophilic than the other tissues, even in emerged cercariae, although all tissues retain their orthochromatic-blue response to the metachromatic dyes. The lessened reaction may be due to the assumption of differential activity by the cercarial tissues. It possibly results from a concurrent dilution of their cytoplasm.

As the anterior group of glands discharge, a series of small accumulations of mucoid remain in the curved, distal portions of their ducts (Figs. 5, 6). These reservoirs form in all of the opisthorchoid cercariae studied. They are located along the border of a slight pit or depression immediately anterior to the mouth of each cercaria (Figs. 5, 7). The pit is connected to the mouth by a shallow trough.

The discharged mucoid from the glands forms a thin film over the entire bodies of the individual cercariae. This film is highly metachromatic to toluidine blue and thionin following either basic lead acetate or Bouin's fixation. It persists in its entirety for the remainder of the stay in the snail tissues. In the available preparations of normally emerged opisthorchoid cercariae, however, the only demonstrable remnant of the peripheral mucoid envelope is a faint metachromatic-red coloration around their anterior ends. This evidence of mucoid or mucopolysaccharide substances is difficult to demonstrate. Possibly the mucoid film has become more delicate through loss of portions of its substance and can be demonstrated only under less rigorous conditions of technique than those necessary for the preparation of whole mounts. The persistent metachromatic reaction at the anterior ends of the cercariae indicates, however, that significant accumulations of mucoid substances have been adequately demonstrated in these cercariae. The possibility remains that the film is retained by the emerged cercaria but that its fundamental nature is so changed that it is no longer metachromatic. However, any general mucoid film must be much more delicate than similar films described for xiphidio-cercariae (Kruidenier, 1951, 1953).

The accumulations of mucoid trapped in the terminal portions of the ducts from the anterior glands persist in the freshly emerged cercariae. They become much more dilute in older specimens indicating a continued discharge after the emergence of the cercariae. This discharge may account for the persistent metachromatic reaction at the anterior ends of the cercariae. Whether or not these remnant mucoids are of any value to the cercariae is speculative.

#### DISCUSSION

Mucoid discharge and the formation of an enclosing envelope preceding cercarial migration through host tissues favor the hypothesis that certain mechanical advantages such as lubrication of their migratory movements are derived from the mucoid substance. Analysis of the host-parasite relations of the cercariae indicates further probable function for the transitory peripheral mucoid film.

The details of cercarial development are essentially parallel in the species of opisthorchoids studied. Primary differentiation is intra-redial but a very considerable portion of their development and growth occurs, adjacent to the rediae, in the tissues of the snail hosts. The degree of final development attained within the snail is characteristic of individual species and is uniform. No evidence of precocity within the snail is apparent in the available material. The descriptions of the cercariae of *Euryhelminis monorchis* and *Caecicola parvulus* (Ameel, 1938, Lundahl, 1941) do not indicate any variation in their finally attained differentia-

tion. Ranges reported in body measurements are explainable on the basis of the variable contraction of different individuals. Cercariae, as individuals, develop into adult parasites. They are thus unquestionably capable of much more extensive growth and development than occurs in the snail host. The uniform morphology of the emerged cercariae implies a precise, rigorous control of the extent to which they may develop within the snail. They must reach other hosts to realize their full potential. Morphological variation would certainly occur if the suspension of their developmental processes were dependent on their time of emergence from the snail. It is highly improbable that the very numerous cercariae would remain in the snail for the identical periods that their uniformity would necessitate. So far as is known cercarial emergence depends on the activities of each individual.

That the presence of alien organisms in their tissues stimulates inimical activity on the part of host organisms has become axiomatic. It is difficult to see how the invasion of the snail by parasites could fail to so stimulate the snails. The immunity of different species of snails to specific infections indicates such a general reaction on their part. The duration of the usual infection of snails by larval trematodes allows ample time and stimulus for the snail to produce immunizing factors.

During their primary development the opisthorchoids are possibly protected by the rediae in which they develop. Immature cercariae, emergent from their rediae, are not so protected from inimical host activities. Their growth and development can only be at the expense of and despite the host. Such developing cercariae must be very well adapted. A change from excellent adaptation to the snails to an adaptation for their next host, and to free existence, must occur during their sojourn in the snail as cercariae are capable of penetration into the second intermediate host upon leaving the snail. The changed adaptation must occur during the final maturation period of the cercariae. The discharge of their mucoid glands and the formation of the mucoid envelope appear to initiate the final maturational changes of the cercariae. The mucoid film interposes an extra layer of material between the cercariae and their environment. It is difficult to see how such a coating can fail to create a protective barrier against an environment which might become more hostile as intrinsic changes occur in the parasite.

Such a barrier might also interfere with the ability of the cercariae to make adequate use of the host. For example it might lessen their ability to obtain essential food materials from the adjacent tissues. The mucoid coat could thus stimulate emergence activities on the part of the cercariae.

Many mucopolysaccharides and mucoids are effective inhibitors of proteolytic enzymes (Meyer, 1945). The value of such a coating substance to parasites completing their stay in the digestive glands of their hosts seems obvious. The defensive nature of the capsular mucopolysaccharides of numerous pathogenic bacteria suggests very strongly a similar protective function for the mucoid envelope which is formed around the opisthorchoid cercariae. Comparatively less mucoid is produced by the opisthorchoids than by other cercariae reported (Kruidenier, 1947, 1951, 1953).

#### SUMMARY

The development and morphology of a series of ventral mucoid glands is described in four species of opisthorchoid cercariae. The glands discharge as the

cercariae approach maturity. Their metachromatic contents form complete envelopes around the bodies of the cercariae. Only a portion of the envelope is demonstrable on normally emerged cercariae, strongly indicating the use of the mucoid during the cercarial sojourn in snail hosts. The mucoids may have mechanical and physiological functions.

As in xiphidiocercariae (Kruidenier, 1951, 1953), metachromasy within the differentiating penetration glands indicates that mucoid and penetration glands develop from a common cell type.

Insufficient data make it impossible to determine the possible taxonomic value of the mucoid glands in various groups of cercariae. The present investigation demonstrates that such glands probably do not form a basis for the separation of opisthorchoid groups.

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#### EXPLANATION OF FIGURES

All drawings were made with the aid of a camera lucida. One unit of scale indicates a magnification of 0.01 mm. Figs. 1-4, 8-9 are drawn to the same scale. Specimens for figs. 6, 7 were fixed in corrosive sublimate and stained in thionin, those for figs. 8, 9 were Bouin's, toluidine blue preparations. The remainder of the specimens were basic lead acetate, toluidine blue preparations.

Abbreviations used: ac.—acetabulum; d.—duct; ep.—eye spot; mg.—mucoid gland; pg.—penetration gland; pi.—pigment; te.—enlarged duct from mucoid gland; rf.—reproductive fundament.

FIGS. 1. and 2. Stages in the development of *Euryhormis monorchis*

FIGS. 3. and 4. Developmental stages, cercaria from *Ammicula limosa*

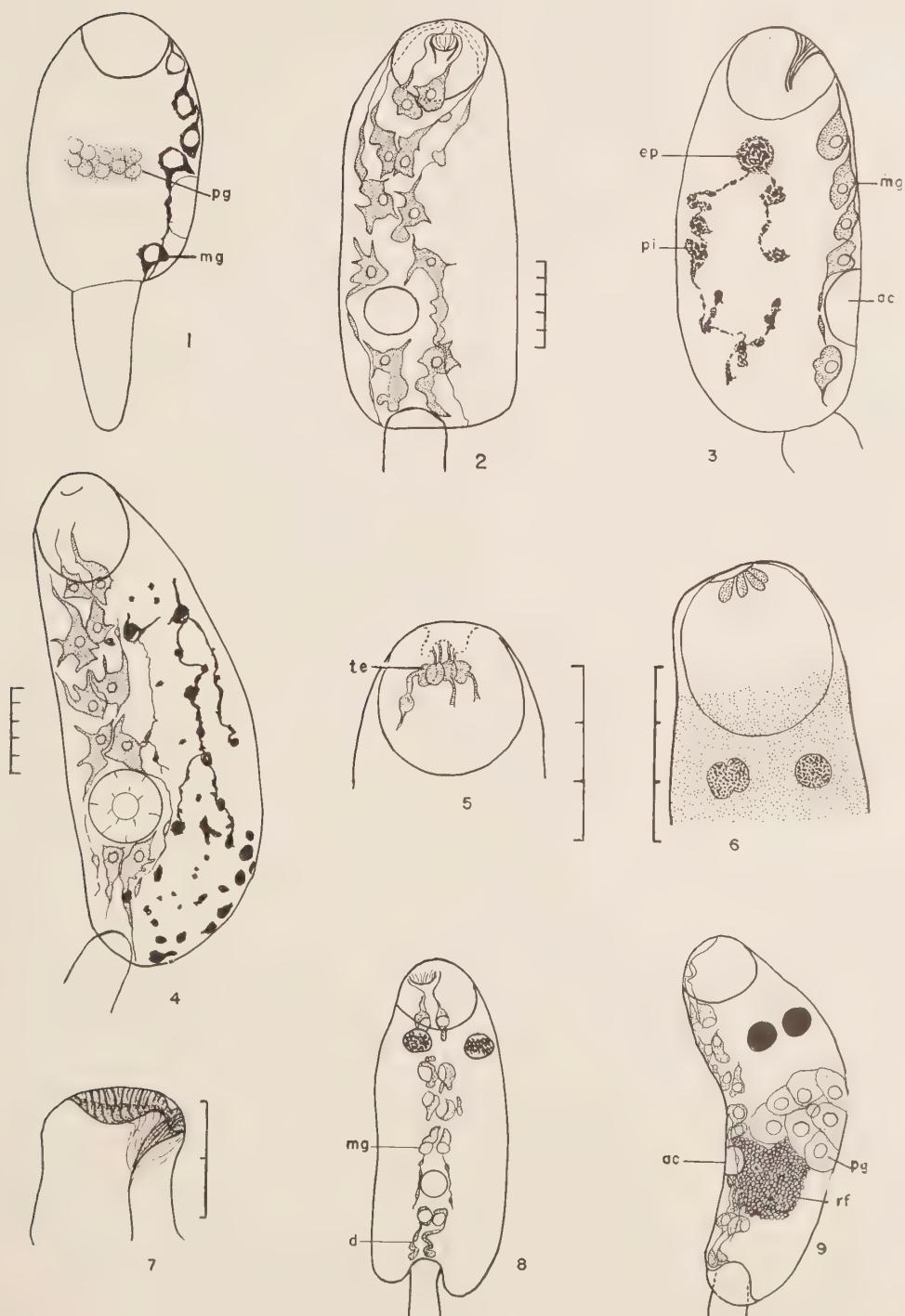
FIG. 5. Anterior end of pre-emergent cercaria from *A. limosa*.

FIG. 6. Anterior end of emerged cercaria from *A. limosa*

FIG. 7. Anterior end of emerged cercaria (from *A. limosa*) showing mucoid-coated anterior pit and buccal cavity.

FIGS. 8. and 9. Developing stages, *Caecicola parvulus*.

PLATE I



# A NEW GENUS AND SPECIES, *FLORIDOSENTIS ELONGATUS*, OF NEOECHINORHYNCHIDAE (ACANTHOCEPHALA)<sup>1</sup>

HELEN L. WARD

The University of Tennessee, Knoxville, Tennessee

During the month of August, 1952, the viscera of ten mullets, *Mugil cephalus*, caught in Biscayne Bay off Miami, Florida, were examined for helminth parasites. Twelve acanthocephalans belonging to the family NEOECHINORHYNCHIDAE were collected from the intestines of five of these fish; not more than two or three worms were found in each host. Since these parasites differ fundamentally from other genera of the family, a new genus, *Floridosentis*, is here proposed with *F. elongatus* as the genotype. Bangham (1938, 1940) examined fresh-water fishes of southern Florida and found *Leptorhynchoides thecatus* and *Neoechinorhynchus cylindratus* in several fishes of the family Centrarchidae, but in six mullets examined he found no acanthocephalans. The members of the family NEOECHINORHYNCHIDAE are chiefly parasites of fresh-water fishes, but Chandler (1935) reported *Atactorhynchus verecundus* from Galveston Bay, and many other species have been reported occasionally from marine fishes. Since the mullet is a mud-feeder, it is possible that the immature stages of the parasite described in this paper occur in a crustacean living in the mud of rivers or estuaries.

## *Floridosentis*, new genus

**Diagnosis:** With the characters of the family Neoechinorhynchidae. Body long and slender, nearly uniform in diameter, slightly enlarged in anterior region. Proboscis cylindrical to club-shaped with eight diagonally longitudinal rows of approximately seven hooks in each row. The anterior hooks are large and provided with conspicuous roots and there is a gradual decrease in the size of the hooks posteriorly. Lemnisci very long and broad, one with two nuclei, the other with one. Parasitic in intestine of fishes. Development unknown.

**Genotype:** *Floridosentis elongatus*, n. sp.

## *Floridosentis elongatus*, new species

Figs. 1-3

**Description:** With the characters of the genus *Floridosentis* as given above. Females (Fig. 1), not fully mature, up to 25 mm. in length and 1 mm. in maximum diameter, about 0.75 mm. in diameter throughout most of body length. Males (Fig. 3) up to 18 mm. in length and 1 mm. in maximum diameter. Body wall about 0.05 mm. thick. Proboscis (Fig. 2) 0.30 to 0.40 mm. long and 0.18 to 0.20 mm. in maximum diameter. Proboscis hooks not in perfect longitudinal and circular rows, but arranged in eight diagonally longitudinal rows of approximately seven hooks in each row. Anterior hooks 0.040 to 0.050 mm. in length with rectangular roots slightly longer than the hooks. Hooks in mid-region of proboscis about 0.034 mm. in length and very slender. Basal hooks very small, 0.013 to 0.020 mm. long. Apical organ of proboscis very large and protruding from tip of proboscis in some specimens. Neck very short, 0.05 to 0.10 mm. in length. Proboscis receptacle up to 0.60 by 0.18 mm. in males and 2 by 0.20 mm. in females. Lemnisci about half the length of the body and about 0.20 mm. in width. Binucleate lemniscus slightly longer than uninucleate lemniscus. The longitudinal lacunar vessels of the lemnisci are very conspicuous. Male genital organs occupy posterior half of body. Testes nearly equal in size, each measuring about 2.20 by 0.50 mm. The syncytial cement gland contains eight nuclei and is slightly larger than a testis. Cement receptacle ovoid, about 0.60 by 0.40 mm. Body cavity of females filled with oval-shaped ovarian balls

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measuring about 0.15 by 0.10 mm. and elongate embryos measuring about 0.034 by 0.006 mm. Embryos not provided with the outermost shell.

*Definitive host:* *Mugil cephalus*, in intestine.

*Locality:* Biscayne Bay, Florida.

*Type material:* Holotype male, U. S. N. M. Helm. Coll. No. 37389.

Allotype female and paratypes representing both sexes in Parasitology Collection, Department of Zoology and Entomology, University of Tennessee, Knoxville.

The genus *Floridosentis* differs from the other genera of the family NEOECHINORHYNCHIDAE chiefly in the number and arrangement of proboscis hooks. The genera *Neoechinorhynchus*, *Octospinifer*, *Eocollis*, and *Gracilisentis* are all characterized by the possession of a proboscis with three circular rows of hooks. In the genus *Atactorhynchus* there are eight diagonally transverse rows of hooks, the first 4 or 5 containing 8 hooks each and the last rows containing about 16 hooks each; only the tips of the hooks project through the cuticle. The proboscis of *Tanaorhamphus* is relatively longer and provided with many more hooks than that of *Floridosentis*. The genus *Paulisentis* is characterized by a small proboscis with six diagonal rows of five hooks each. Van Cleave and Bangham (1949) have given a discussion of speciation in the family NEOECHINORHYNCHIDAE and have pointed out the parallel in evolutionary progress of the parasites of this family and their hosts.

I wish to express my appreciation to Dr. F. G. Walton Smith for use of the facilities of the University of Miami Marine Laboratory while engaged in collecting parasites from fish caught in the Miami area, and to Dr. H. J. Van Cleave for confirming my opinion that the parasites described here represent a new genus.

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#### EXPLANATION OF PLATE

All figures are of *Floridosentis elongatus*, n. g. and sp.

All drawings made from stained whole mounts.

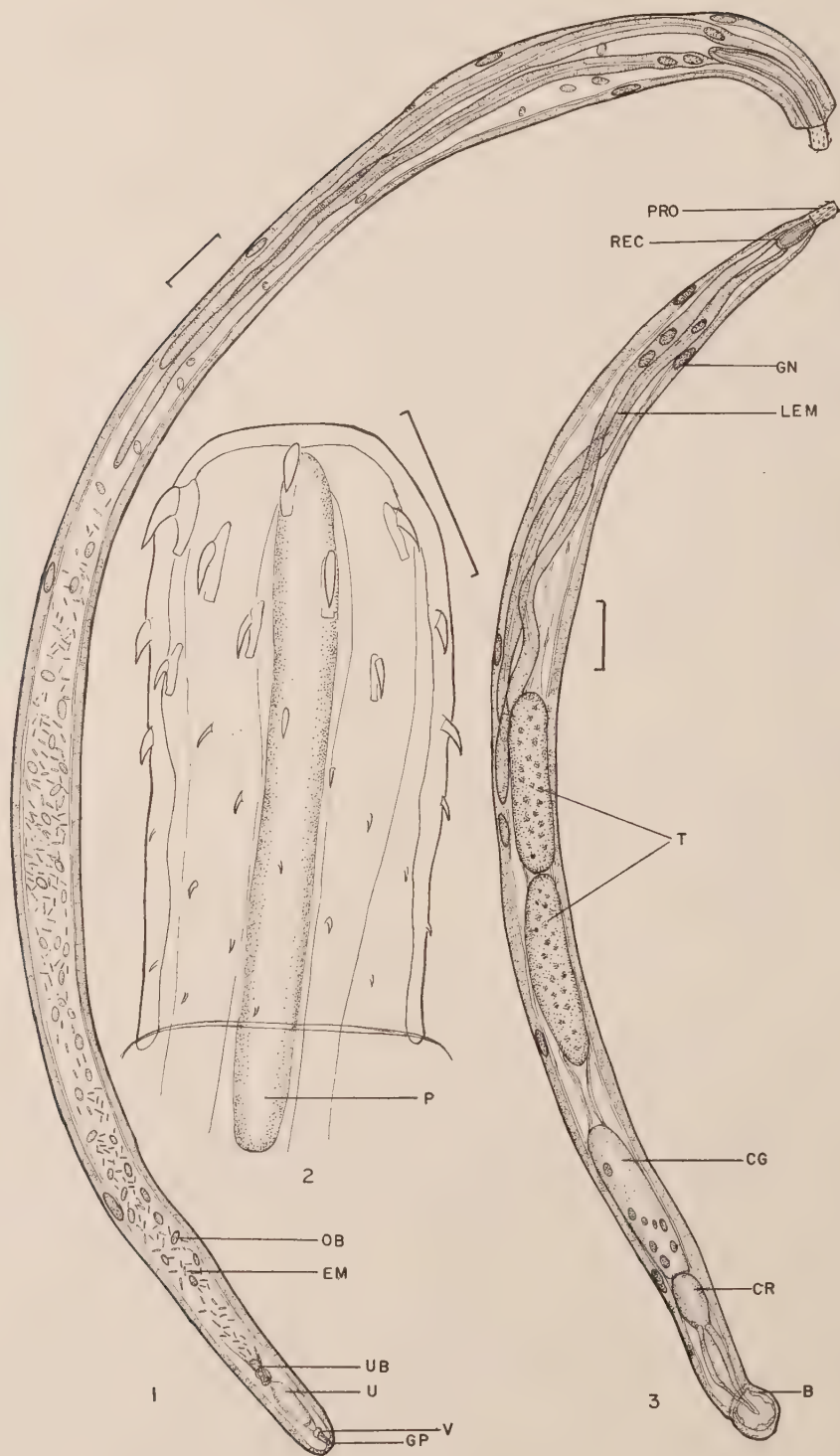
FIG. 1. Adult female, drawn from microprojection. Scale has value of 1 mm.

FIG. 2. Proboscis, camera lucida drawing. Scale has value of 0.1 mm.

FIG. 3. Adult male, drawn from microprojection. Scale has value of 1 mm.

Abbreviations used: B, bursa; CG, cement gland; CR, cement receptacle; EM, embryo; GN, giant nucleus; GP, genital pore; LEM, lemniscus; OB, ovarian ball; P, apical organ; PRO, proboscis; REC, proboscis receptacle; T, testes; U, uterus; UB, uterine bell; V, vagina.

## PLATE I



A SUBCUTANEOUS, CYST-PARASITE OF BULLFROGS:  
*HISTOCYSTIDIUM RANAE*, N. G., N. SP.

CHAUNCEY G. GOODCHILD

Department of Biology, Emory University  
Emory University, Georgia

INTRODUCTION

Peculiar cyst-forming parasites of vertebrates have been reported periodically since 1907 from Europe and the Americas. Five genera containing twelve or more species from poikilothermic and homoiothermic hosts have often been included in this group of enigmatic parasites. *Dermocystidium pusula* occurs as small, wart-like swellings in the skin of several European urodeles (Pérez, 1907, 1913; Moral, 1913; Guyénot and Naville, 1922; Gambier, 1924). Three species of *Dermocystidium*, forming tiny, whitish pustules in the gills have been reported from European and North American fish: *D. branchialis* from *Trutta fario* (Léger, 1914; Dunkerly, 1914), *D. vejdoskyi* from *Esox lucius* (Jírovec, 1939), and *D. salmonis* from *Oncorhynchus tshawytscha* (Davis, 1947). A single species, *D. ranae*, occurs as U-shaped cysts in the skin of several European frogs (Guyénot and Naville, 1922; Remy, 1931; Henneguy, in Pérez, 1913; Broz, 1944; Broz and Privora, 1952). All species in the genus produce spores characterized by a large, refractive inclusion body; they are specifically distinguished by the shape of the cyst, its location in the host, and by sporic differences. The genus *Dermosporidium* contains three species also forming cysts in frogs and fishes. *Dermosporidium hylarum* has been reported from South American *Hyla rubra* (Carini, 1940), and *D. granulorum* in *Rana temporaria* from Czechoslovakia (Broz and Privora, 1952). A single species, *D. trutta*, occurs as a gill parasite in *Trutta fario* (Weiser, 1949). Species in this genus have multiple inclusion bodies in the ripe spore rather than a single one as in *Dermocystidium*. Two additional genera of these cystic parasites, *Dermomycoides* and *Hepatosphaera*, have been reported from urodeles. The former was erected by splitting from *Dermocystidium* organisms having flagella but with no inclusions in their spores. *Dermomycoides* at present contains two species of newt parasites, *D. beccari* (Granata, 1919), and *D. armoriacus* (Poisson, 1937). *Hepatosphaera molgarum* is a grave hepatic parasite of newts which in the process of sporulation causes liquefaction of the infected organ (Gambier, 1924). Finally, there is an extremely interesting, widely distributed parasitic genus, *Rhinosporidium*, occurring in bovines and equines and with a single species, *R. seeberi*, in man. Species of *Rhinosporidium* induce polypoid hyperplasia of connective tissues of nasopharynx, conjunctiva, lacrimal sac, ear and penis (Wright, 1907; Ashworth, 1923; Elles, 1941; *et al.*).

Because of the absence of ontogenetic data, it is problematical whether these forms are all lower fungi, or whether some may not be simple protozoans. Furthermore, it is practically certain that several unrelated forms have been included in this group merely because of identical host preference or superficial resemblances.

In the course of an investigation on gorgoderid trematodes of frogs (Goodchild,

1950) five small *Rana catesbeiana* were discovered, on September 1, 1949, which were conspicuously swollen in the rump with a tightly-packed mass of minute cysts. Although the uniqueness of the find was recognized, the primary objective prevented investigation of this new form at the time. These frogs, as well as nineteen others, had been taken by a professional collector that day from one of the numerous fresh-water ponds near Woods Hole, Massachusetts; unfortunately the exact pond was not noted on the collection tag, and although recent collections have been made in suspected ponds during the summers of 1950 and 1951, the parasite has not been encountered again. All information presented here accordingly has been gleaned from the preserved specimens and from brief observations made in life. The name *Histocystidium ranae*, n.g., n.sp., is proposed for the parasite.

#### MATERIALS AND METHODS

The infected animals were fixed in either Bouin's fluid (2 specimens) or in 10 per cent formalin (3 specimens). To facilitate rapid penetration of the fixative and to insure preservation of adequate cytological details the skin was cut and reflected in those specimens placed in Bouin's fluid.

Whole mounts were prepared by teasing specimens free from the mass and staining them with paracarmine and iron alum hematoxylin. Thin sections ( $7\ \mu$ ) and thick ones (up to  $50\ \mu$ ) were cut from blocks embedded after the usual dehydrating procedures. These were treated with a variety of reagents including Harris' and Heidenhain's iron alum hematoxylin with and without eosin to determine the nature of the cyst wall, types of cellular inclusions and the nuclear details, as well as the nature of the cellular reactions of the host.

Photographs of preserved hosts in dorsal and lateral views (Plate 1) were made before the frogs were cut up for the preparation of whole mounts and sections of infected tissue.

#### OBSERVATIONS

All five infected frogs were remarkably uniform in size, measuring 60–62 mm. from snout to rump, but were strikingly different in appearance and behavior from uninfected ones of the same size. If the animals had been smaller one might have thought, at first glance, that the lumps were merely stumps of degenerating tails (Figs. 1, 2, 3, Plate 1). Normal frogs released in a small wire container jumped and climbed in their efforts to escape; infected ones appeared listless and hunched quietly on the bottom of the cage. Furthermore, there seemed to be positive correlation between their degree of apathy and the size of their lump.

In life, the mass was a purplish-red color and protruded posteriorly from the region of the sacrum. In the oldest infections the anal aperture and the posterior surface of the thighs were overgrown by the mass which also showed slight necrosis and ulceration posterodorsally. The smallest growth (Fig. 1 Plate 1) was 16 mm. wide at the posterior tip of the urostyle which jutted 1 mm. from the middle of a dark transverse ridge. Next in size was a lump 17 mm. long and 15 mm. wide (Fig. 3 Plate 1) in which the urostyle was deeply buried. The largest protuberance extended posteriorly 26 mm. from the sacrum and was 19 mm. wide at the level of the hidden anal opening (Fig. 2 Plate 1).

After fixation in formalin the posterior lump was still darker than the remainder of the body. Fluid had either diffused into the subcutaneous area or part

of the dorsal lymph space had been pushed posteriorly during enlargement of the parasitic mass, because the skin had the usual looseness evident in frogs preserved in formalin, and furthermore, when reflected from the lump it appeared normal in color. The darkness was thus caused by the deeper-lying, compact parasitic mass. Grossly the body was otherwise normal in appearance. Bouin's fluid stained the whole organism so yellow that delicate differences in color were lost.

When the skin was reflected from the underlying parts the real extent of the infection was manifest (Figs. 4, 5, 6, Plate 1). Anchored at the sacrum and swelling posteriad there was a brownish mass sharply delimited from the more anterior, whitish longissimus dorsi and latissimus dorsi muscles. Anteriorly the parasitic mass bifurcated, proceeded forward between the coccygeosacralis muscle and the ilium and disappeared, lateral to the longissimus dorsi, beneath the latissimus dorsi. Many chromatophores, apparently melanophores, concentrated on the lump contributed to the dark color noted previously. However, the main content of the mass itself was a light brown color conspicuously darker than the tissues of the host. The mass was also traversed superficially by fine nerves running laterad to the skin. A few blood vessels, strikingly bordered by chromatophores, were also visible on the surface. The outer surface of the lump was smooth and compact; it was a membranous layer consisting of fibrous elements.

Observed under the binocular dissecting microscope the entire surface of the frog appeared peppered with small pustules, which ranged up to 0.3 mm. in diameter, at the summit of slight elevations. Frogs with the largest lumps had the most numerous and largest pustules. The opalescent spherules, actually minute cysts identical to those within the main mass, were numerous on the dorsum and the hind legs; they were less abundant on the forelegs and seemingly absent (at least not visible as projections) ventrally. In a heavily infected specimen random average counts of areas 2 mm. square gave the following distribution: 7 cysts on the dorsal surface, 12 cysts on the hind legs, 5 cysts on the forelegs. This same specimen had also, on the surface, flattened cysts and tiny depressed areas which suggested that former cysts had either been lost *in toto* or had ruptured to discharge their contents.

Cysts were not limited to the massive posterior lump or to the surface of the skin. Rather they occurred in fascial, perimysial, and other connective tissue zones elsewhere in the body, particularly dorsally and laterally where they often formed small islands of one or more cysts. Often they were arranged in linear groups lying parallel to the muscle cells. They were, even with magnifications available on the binocular dissecting microscope, not visible ventrally or among the viscera.

In order to prepare sections, blocks of tissue were cut from the host. Cleanly cut surfaces revealed enormous numbers of cysts. Some of the cysts were milky and opaque, others were almost transparent. Between the cysts, binding them firmly together, was a fibrous stroma of host origin. Many blood vessels, filled with corpuscles, were seen in the interior of the mass.

Stained sections studied with the compound microscope revealed how enigmatic is this parasite. On the basis of revealed structure it is easy to comprehend the taxonomic indecisions of earlier workers regarding organisms of this type. Surrounding the whole lump externally was a thin, fibrous membrane, 0.014–0.032 mm. thick, consisting of layers of fibers or plates which in permanent preparations were

often frayed and wavy. Embedded in the membrane were elongated, darkly-staining bodies, 0.018–0.128 mm. long by 0.003 mm. wide. Scattered throughout the section were innumerable spherical cysts, ranging in diameter from 0.02–0.30 mm., set in a fibrous and cellular ground substance which formed a compact layer approximately 0.003–0.014 mm. thick around each cyst (Fig. 1 Plate 2). Between the cysts the stroma was more diffuse and myxomatous, but revealed fibroblasts, histiocytes, leucocytes, and melanophores; adjacent cysts often showed a confluence of outer fibers which were shared by both cysts (Fig. 1 Plate 2). Immediately subjacent to the compact fibroblastic layer was the true cyst wall, 0.024 mm. thick, composed of outer and inner layers of nearly the same thickness. The outer layer, averaging about 0.01 mm. thick, which was undoubtedly produced by the host, was heavily chromophilic and deeply corrugated. This sulcus-gyrus like feature, which may be a shrinkage artifact, was vividly apparent in tangentially cut sections (Fig. 1 Plate 2). From beneath, the hollows of the gyri were filled with a homogenous material which also constituted an innermost surface of the outer layer. Certain cysts had peculiar areas in the compact outer fibrous envelope. In these there were interruptions where the layer appeared frayed or unraveled (Fig. 2 Plate 2). In no case was there observed a true sporangial or cyst pore as occurs in *Rhinosporidium seeberi*, but these thinner areas may be points of weakness which rupture to discharge the contents of the ripe cyst. The inner chromophobic layer, also about 0.014 mm. thick, which was secreted by the parasite was hyaline, but possessed concentric striae.

Within the cavity of the cyst and filling it completely was a vacuolated syncytium having the form of a chromophilic reticulum (Fig. 2 Plate 2). In optical section the periphery, 0.003 mm. thick, was uniformly continuous but had inner tags of protoplasm joined to the central syncytium. A most unusual feature of this granular reticulum was the presence, in many cysts, of strands irregular in outline and widely spaced, while in others, they were practically filiform and tightly packed (Fig. 6 Plate 2). Surprising also was the lack of correlation between the size of the cyst and the type of reticulum; both types of networks, as well as intermediates, occurred in cysts of all sizes. The significance of this inconsistency is not known. Numerous rather ill-defined and chromatically refractory bodies located in the centers of ameboid-like bits of cytoplasm probably represent nuclei (Figs. 3, 5 Plate 2). They are approximately 0.003 mm. in diameter and are slightly more transparent than the adjacent cytoplasm. Centrally each contains an endosome about  $1\ \mu$  in diameter; radiating from it are delicate strands which insert on the nuclear membrane also provided with fine chromatin-like granules. In certain cysts there are cytoplasmic vacuoles, lying in contact with the nuclear membrane, which form a vacuolated halo about the nucleus (Fig. 3 Plate 2). If the darker reticulum be considered the continuous phase, the lighter discontinuous phase appears hollow or vacuolated; suspended in this vacuole there are in most cysts, spherical to rod-shaped concretions ranging from visible limits to 0.006 mm. in diameter.

Other stages observed but not positively correlated to any developmental sequence of the organism include a very diffuse strand-like organization in which the filaments tend to cluster around the many nuclei (Figs. 4, 6 Plate 2), and a uniform distribution with the cyst lumen of tiny ovoid or spherical chromophobic

objects, 1–2  $\mu$  in diameter, which may represent spores (Fig. 7 Plate 2). The latter stage, which may be the last one in the frog host, also revealed several elongate crystals in the cyst cavity.

#### DISCUSSION

The several species usually considered in reviews on this assemblage of organisms are apparently not closely related. Various authors have speculated that they may lie between the animal and vegetable kingdoms (Moral, 1913; Weiser, 1949), that they may have close affinities to *Blastocystis* (Alexeieff, 1911; Grassé, 1926), or that they may be Haplosporidia, itself a poorly defined group (Léger, 1914). Most authors have suggested that some are lower fungi, in or close to the family Olpidiaceae of the Chytridiales, a member of the Archimycetes section of the class Phycomycetes (de Beauchamp, 1914; Ashworth, 1923; Ciferri, 1932; Gwynne-Vaughan, and Barnes, 1937; Poisson, 1936, 1937; *et al.*).

Experimental life history studies have been largely fruitless. Ripe spores injected by several workers (cf. Poisson, 1937) into the same species of host failed to establish a new infection. Because of this failure, Pérez (1913), and Broz and Privora (1952), and others, have postulated the existence of unknown intermediate hosts. That these vector hosts may be invertebrates is suggested by the infection of cladocerans with a *Dermocystidium*-like parasite, *Lymphocystidium daphniae*, reported by Rühberg (1933), and Jírovec (1939). Swimming zoöspores of *Dermomycoides* are thought to penetrate the skin of its host directly according to Poisson (1937). *Rhinosporidium seeberi* may proliferate vegetatively for years in man. Its method of transfer from man to man are unknown, but propagation within the host results from germination along lymph tracts of spores discharged from ripe sporangia. Ripe spores injected subcutaneously into mice, rats, rabbits, guinea pigs, and monkeys failed to establish an infection, while spores suspended in various media *in vitro* either failed to germinate or succumbed (cf. Ashworth, 1923). Weller and Riker (1930), reviewing the occupational activities of persons infected with *R. seeberi* pointed out the large number who are farmers, tanners, or otherwise intimately associated with animals or their products. Countries such as India and Iran where bovine and equine draft animals are widely used show high infection rates. It is entirely possible that man is merely an occasional and accidental victim; fortunately there is little danger of spread from man to man. Although naturally ruptured cysts were not positively identified in *H. ranae*, vegetative propagation must exist to produce the enormous numbers of cysts occurring in heavily infected frogs. It is entirely possible that ameboid stages from germinated spores migrate via lymph channels or tissue fluid spaces from deeper centers to the skin and other regions. Abundant food materials in the tail and tail base area during the resorption of the tadpole's tail might also contribute to the localization and proliferation of the parasites in that area.

*Histocystidium ranae* resembles *Rhinosporidium seeberi* in the nature of the cyst wall, the fat and protein-like granules in the protoplasm, and the histoplasia which they both elicit; moreover, it resembles *Dermocystidium vej dovskyi* and *D. pusula* in the character of the vacuolated syncytium. Both *H. ranae* and *R. seeberi* have double envelopes about their sporangia in addition to an encircling layer of host fibers; Beattie (1906) stated that larger cysts of the latter species

have thinner walls, a condition not true for *Histocystidium*. The two species are not congeneric because *Rhinosporidium* initially has a mononucleate thallus and later has an elevated sporangial pore through which ripe spores are discharged, features not present in *Histocystidium*. As noted above, however, the protoplasm of *H. ranae* contains rods and spherules which stain with Heidenhain's hematoxylin; in form and position they resemble concretions found in *R. seeberi* and said, by Ashworth (1923), to be fat and protein reserves. A marked histoplasia of fibroblasts and fibers, histiocytes, and infiltrated polymorphonuclear leucocytes, with local dense cellular aggregations in a more extensive myxomatous matrix is a common host reaction to infection with these two species. Between *Histocystidium* and *Dermocystidium* there are other differences. The latter produces a cyst wall which is uniformly hyaline inside. There are also differences in the syncytium which in the former has a marginal layer not found in the latter, according to Dunkerly (1914). Differences in the size of the cysts and their diffuseness or agglomeration into clumps serve also to differentiate genera in this assemblage of organisms. Both species of *Dermomycooides* occur as non-clumping, wide-spaced subcutaneous cysts; hyperplasia is relatively absent and the cyst content is centro-peripherally differentiated, characters in sharp contrast to *Histocystidium*. The other possibly related form, *Hepatosphaera molgarum*, is an internal parasite with histolytic effects.

Although the material available for *H. ranae* did not reveal spores similar to those described in other species, in all probability the tiny objects shown in Fig. 7 Plate 2 constitute the dispersal phase and are eventually extruded to the outside by ulceration of the overlying skin. In other species spores are so characteristic in form and content that they are of great taxonomic use. In these other species, as the plasmodial pansporoblast matures, small multinucleate areas are compartmentalized by condensation of septa from cytoplasm. Within the chambers uninucleate spores develop, with concurrent formation from tiny precursor vacuoles or granules, of a characteristic refractive inclusion body in each spore. In the genus *Dermomycooides* each ripe spore bears a long flagellum which begins to lash when the spore is discharged into water.

In all probability the effects of these parasites on their hosts depend upon their position in the body, alteration in organization of tissue, and the presence or absence of vegetative propagation in the host. Skin parasites and gill parasites which do not continue vegetative propagation within the host are reported to be relatively benign (Pérez, 1913). Certainly dangers of bacterial and other fungal infections are possible in the ulcers produced by sporulation, notwithstanding the prompt infiltration of host phagocytes into the area reported for species of *Dermocystidium* (Broz and Privora, 1952; *et al.*). Interesting, in this connection, is the preference of the anal region for cyst production in *Dermocystidium ranae* reported by Guyénot and Naville (1922), and in *Dermosporidium granulosum* described by Broz and Privora (1952). The hepatic parasite of European tritons, *Hepatosphaera molgarum*, according to Gambier (1924), caused the death of heavily infected captive *Molge cristata* within two days; these urodeles were sluggish in the field and easily captured. *Rhinosporidium seeberi* causes a persistent infection in man because of continuous self-infection. Patient "M," an Indian medical student, studied by Ashworth (1923), had thirteen nasal operations over

a period of about ten years. Surface polyps which reformed quickly were excised periodically for nine years; finally a heroic operation, which involved removing a large portion of the nasal septum, was performed. There was no assurance that a permanent cure had been achieved and the further history of the patient is not recorded. Wright (1922) reports a cure for Rhinosporidiosis of the conjunctiva by laving three times a day with 2 percent tartar emetic. It is probable that *Histocystidium* likewise propagates vegetatively in the frog and so may perhaps be extremely deleterious in heavy infections.

## SUMMARY

1. Five small bullfrogs (*Rana catesbeiana*) were found to be infected with a large subcutaneous fungoid parasitic mass located posterodorsally, just above the cloacal aperture.

2. The parasitic mass contained thousands of spherical cysts embedded in a stroma of host tissue. Each cyst, ranging up to 0.3 mm. in diameter, was covered with a double wall which consisted of an outer chromophilic fibrous one of host origin and an inner chromophobic hyaline one of parasitic origin. Internally the cysts were filled with a vacuolated syncytial protoplasmic mass.

3. Complete developmental stages were not traced in the limited fixed material available for this study. Several cysts were studied, however, which contained a mass of tiny, 1–2  $\mu$  in diameter, spherical or ovoid objects which possibly were spores.

4. Life history possibilities were discussed as well as a review of other fungoid, cystic parasites occurring in vertebrates.

Abbreviations, used in the illustrations: c, capillary; ci, cytoplasmic isthmus; cr, crystal; cv, cytoplasmic vacuole; f, fibers; fb, fibroblast; fe, fibrous envelope of cyst; h, hyaline layer of cyst; hr, hyaline ring; n, nucleus; pc, peripheral cytoplasm; pv, perinuclear vacuole; s, host stroma; sb, "spore bodies"; sc, syncytial cytoplasm; st, cyst wall cut subtangentially; t, cyst wall cut tangentially; v, cyst vacuole.

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## EXPLANATION OF PLATE 1

FIG. 1. Earliest infection, formalin specimen. Urostyle region is 16 mm. wide, somewhat darker in color and more sharply delimited than in uninfected frog of same size.

FIG. 2. Oldest infection, formalin specimen. Parasitic mass is 26 mm. long by 19 mm. wide.

FIG. 3. Intermediate infection, formalin specimen. Parasitic mass is 17 mm. long by 15 mm. wide.

FIG. 4. Lateral view of specimen fixed in Bouin's fluid. Parasitic mass is 22 mm. long by 16 mm. wide.

FIG. 5. Dorso-lateral view of specimen fixed in Bouin's fluid. Parasitic mass is 20 mm. long by 17 mm. wide. Pigmentation and nerves are apparent on original.

FIG. 6. Dorsal view of same specimen shown as Fig. 4. Anterior bifurcation of parasitic mass evident on original.

PLATE I

FIG. 1



FIG. 2



FIG. 3

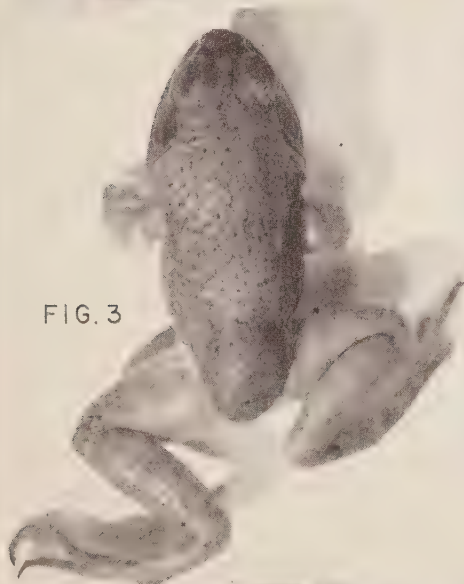


FIG. 4

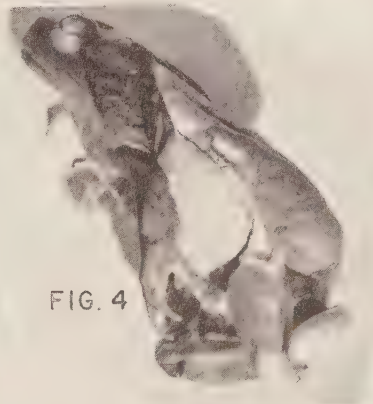


FIG. 5

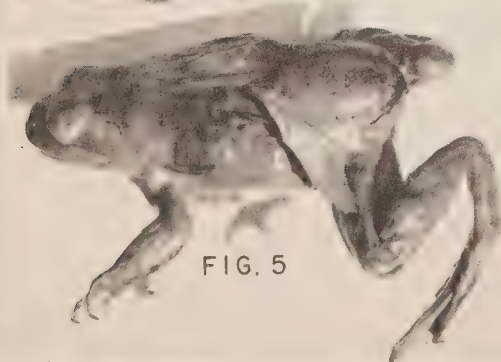


FIG. 6



## EXPLANATION OF PLATE 2

All figures were drawn with the aid of a camera lucida.

FIG. 1. Low power study of a group of cysts with surrounding host stroma.

FIG. 2. Cyst with vacuolated syncytial protoplasm and typical double cyst wall.

FIG. 3. Detail of cytoplasmic island with nucleus and perinuclear vacuolar halo. Typical internal detail of cyst represented as Fig. 2.

FIG. 4. Filamentous stage of cystic development. Nuclei are surrounded by short, rod-like cytoplasmic structures.

FIG. 5. Detail of cytoplasmic island and adjacent marginal cytoplasm from a cyst as illustrated in Fig. 4.

FIG. 6. Stage in which nuclei are more numerous and more refractory to staining. Peculiar hyaline rings similar to the hyaline wall of the cyst are often produced internally.

FIG. 7. Stage in which cyst cavity is occluded with many small "spore bodies," approximately 1-2  $\mu$  in diameter, as well as crystals and large confluent vacuoles.

PLATE 2

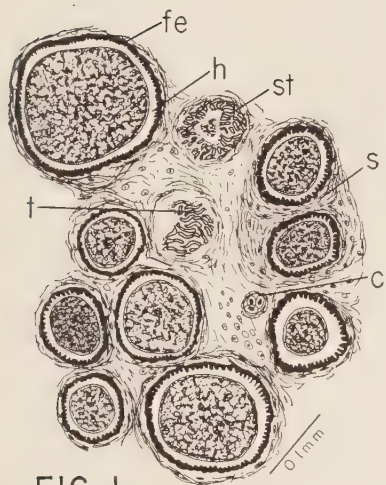


FIG. 1

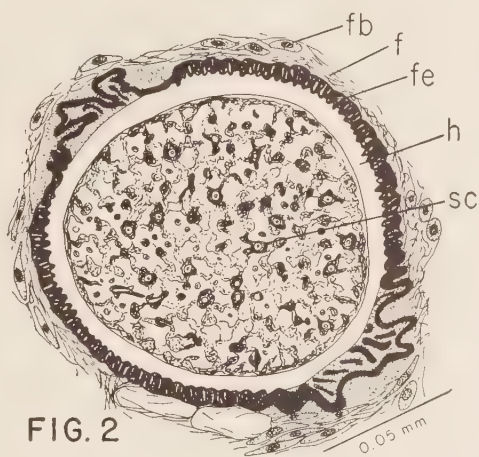


FIG. 2

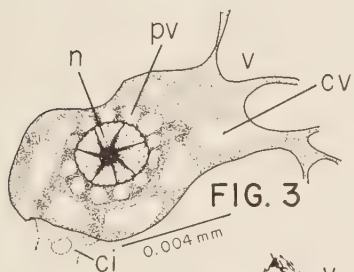


FIG. 3

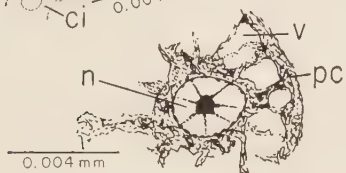


FIG. 5

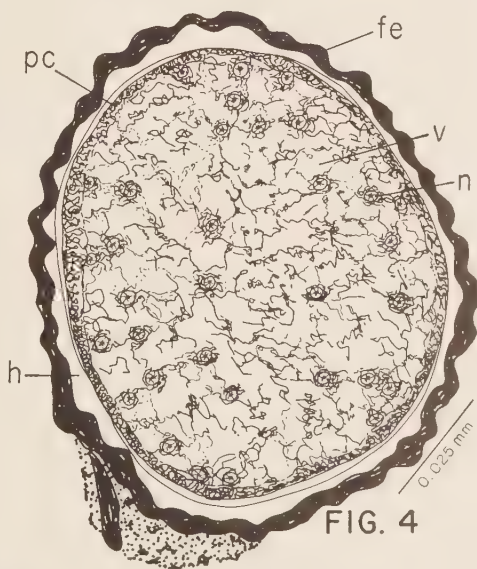


FIG. 4

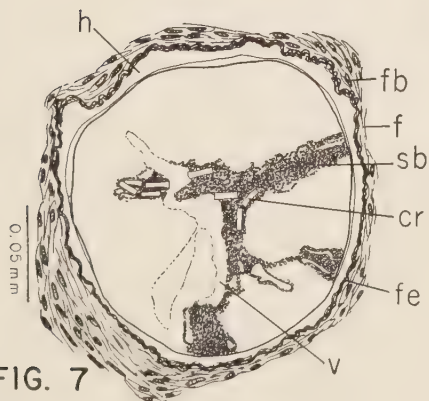


FIG. 7

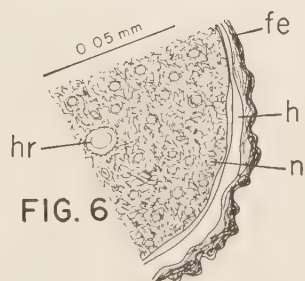


FIG. 6

## TOXOPLASMA IN FROGS\*

REGINALD D. MANWELL, EMIL BERNSTEIN, AND RAYMOND DILLON

Syracuse University, Syracuse, N. Y.

There has been for many years a question as to whether any species of cold-blooded animals either harbor naturally acquired toxoplasmosis, or are experimentally susceptible to it. Splendore (1913) reported the successful infection of frogs (*Cystignatus ocellatus*) with a strain of *Toxoplasma* isolated from the rabbit, and Plimmer (1916) believed he had found a case of toxoplasmosis in a Mexican snake (*Coluber melanoleucus*) which had been in the London Zoological Garden. Unsuccessful attempts to infect frogs (*Rana pipiens*) were made in the senior author's laboratory some years ago, but on too few animals to be conclusive (Manwell, Coulston, Binckley and Jones, 1945). In view of the widespread occurrence of toxoplasmosis in man, and in such animals as the dog, cat, and pigeon, it is of obvious importance to know as much as possible about the host-range of the organism.

For these reasons we have repeated and extended our earlier work, using in all 52 frogs (*Rana pipiens*). Of these, 12 each were inoculated by one of the following routes: oral, intramuscular, and intracerebral. Sixteen others were given the parasites intraperitoneally. The inocula consisted of heavily parasitized peritoneal exudate from acutely infected white mice, and the amount given to each frog varied in different experiments from 0.05 to 0.40 ml. Counts have established the fact that such quantities of exudate usually contain from one to ten million organisms.

The frogs were kept at room temperature in individual battery jars, with separate compartments for water. The first series of 20 animals were checked for infection by the injection into mice of peritoneal aspirate, withdrawn at intervals of 6, 20, 24, 30 and 48 hours and of pooled homogenates of viscera (brain, liver, spleen) into clean mice. Examination of stained organ smears under the microscope was also done after death of the frog. (Frogs so inoculated lived in most cases for about a week, the range in survival time being from 2 to 12 days.)

The second series of animals was treated differently. The 32 frogs of which it consisted, were divided into 4 groups of 8 each, according to the routes of infection, which were the same as listed above. Two of the members of each were then sacrificed after each of the following intervals: 1, 2, 3, and 7 days, and homogenates of liver, spleen, lung, and brain in isotonic saline injected into mice. If these showed no evidence of toxoplasmosis after an observation period of 14 days, a second series of mice was inoculated from the first, and still a third series from them, if the results were still negative.

Several experiments were also done to discover the effect on the parasites of exposure to frog blood. For this purpose peritoneal exudate from acutely affected mice was incubated with whole frog blood at 3° C., and inoculated into clean mice after intervals of varying length. The mice were then followed for the development of toxoplasmosis.

The results of the attempts to infect frogs were again essentially negative. None

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of the animals showed viable organisms after periods longer than 72 hours, and only one of the cases inoculated intracerebrally showed parasite survival for as long as this. Viable organisms could still be demonstrated after 48 hours in intraperitoneally injected frogs, but all the animals given *Toxoplasma* by other routes proved negative. In frogs injected intraperitoneally, peritoneal aspirates withdrawn after a maximum period of 5 hours failed to produce infection in mice. Only in the brain were organisms found in organ smears made after natural death or sacrifice of the animal, nor were there any indications of abnormal symptoms which could be ascribed to the parasites.

Exposure to frog blood apparently had no effect on the organisms, at least for the maximum period of observation of 24 hours, since typical infections resulted in mice from its inoculation.

Thus it appears extremely unlikely that frogs, at least of the species *Rana pipiens*, can harbor *Toxoplasma* for any considerable length of time. It is however of interest that the organisms can survive in the frog brain for at least 3 days. It is of course possible that other strains of *Toxoplasma* (the one used in these experiments was the "RH" strain, of human origin, and originally isolated by Dr. A. B. Sabin) might prove more infectious for frogs, and also that other species of frogs (or of Anura) may be more susceptible.

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THE LIFE CYCLE OF *PARVATREMA BORINQUEÑAE* GEN. ET SP.  
NOV. (TREMATODA: DIGENEA) AND THE SYSTEMATIC  
POSITION OF THE SUBFAMILY GYMNOPHALLINAE

R. M. CABLE<sup>1</sup>

Dept. of Biological Sciences, Purdue University and College of Agriculture and Mechanic Arts,  
University of Puerto Rico

I. INTRODUCTION

There are so many groups of digenetic trematodes for which few or no life histories are known that taxonomic characters must be limited to those of adult stages. Since Looss pioneered in breaking up the old genus *Distomum*, much progress has indeed been made "*zum natürlichen System*" repeatedly emphasized by Odhner in the titles of his papers. Although adult morphology has been of value in taxonomy at the generic and specific levels, life history studies have been more significant in interpreting major interrelationships and phylogenetic lines which make up the framework of a truly natural system of taxonomy and distinguish it from a mere catalog of genera and species.

The morphology and host relationships of adult trematodes have served largely as the basis of existing taxonomic concepts and these schemes have been in part substantially confirmed by life history studies. However, these investigations have also disclosed unexpected affinities between families and demonstrated that some categories proposed on the basis of adult morphology are highly artificial. As a result of convergence and divergence, certain adult structures have proved to be unreliable taxonomic characters above the generic level whereas others, sometimes relegated to a minor role, actually are more constant and hence more significant.

Perhaps no group illustrates these facts better than do the trematodes assigned at one time or another to the family HETEROPHYIDAE. Knowledge of life cycles not only has revealed a close relationship between that family and the OPISTHORCHIIDAE, but also have demonstrated that the HETEROPHYIDAE as formerly constituted was not a natural group. Thus the subfamily MICROPHALLINAE was found to be a distinct family actually more closely related to plagiorchids than heterophyids. When Odhner (1900) erected the genus *Gymnophallus*, he followed Looss in regarding the species assigned to it as being related to the COENOGONIMINAE, which are true heterophyoids. Odhner noted, however, that the COENOGONIMINAE possess a well developed seminal receptacle whereas *Gymnophallus* does not. Since microphallids usually lack that structure, it must have been largely on that basis and the form of the digestive system that *Gymnophallus* was transferred to the MICROPHALLIDAE after that family was erected. In other respects, adults of *Gymnophallus* are more like certain heterophyoids which lack a gonotyl and have a Y-shaped

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excretory vesicle with arms extending into the forebody. These heterophyoids usually are placed in the CRYPTOAGONIMIDAE and ACANTHOSTOM(AT)IDAE which probably should be combined. Markowski (1936) placed *Gymnophallus* in the ACANTHOSTOMIDAE but Price (1940) considered the resemblance of that genus to the heterophyoids to be the result of convergence.

In studies dealing with the MICROPHALLIDAE, neither Baer (1943) nor Cable and Kuns (1951) mentioned the GYMNOPHALLINAE. In the latter study, we were prompted to omit that subfamily because adult structure and especially such information as was then available concerning gymnophalline life histories were incompatible with the well established pattern of the MICROPHALLIDAE. From Pelseneer's (1906) observations on *Cercaria syndosmyae*, a minute furcocercous larva developing in marine bivalves, Markowski (1936) had suggested that the adults of all such cercariae might be species of *Gymnophallus*; Odhner (1911) had referred larvae of this type to the FELLODISTOMATIDAE but without experimental evidence. Lebour (1912) and Rees (1939) had described for a species of *Gymnophallus* the metacercaria and a cercariaeum, both in bivalves. Although certain microphallids have tailless larvae, others do not and the absence of a tail is not in itself an indication of affinity. Furthermore, microphallid larvae develop in gastropods and those with tails encyst in arthropods, usually crustaceans. These differences in the host relationship of immature stages increased our doubt that the GYMNOPHALLINAE are at all closely related to the MICROPHALLIDAE. That doubt now seems to be confirmed by the life cycle of *Parvatrema borinquenae* and observations on forms related to this new genus and species belonging to the GYMNOPHALLINAE. This paper extends the report in a recent abstract (Cable, 1952).

## II. METHODS

Drawings of adult specimens except those copied from other papers and acknowledged elsewhere were made by microprojection of whole mounts fixed in corrosive sublimate-acetic acid and stained with either Semichon's carmine or Harris' hematoxylin. All other figures are free-hand, those to scale being drawn from measurements of specimens killed in hot sea water, mounted without cover glass pressure and used immediately. Details were added from study of living specimens, some of which were stained with neutral red. Figures of two cercariae and one new adult not in the life cycle reported here are included for aid in the interpretation of relationships. These forms are described merely to the extent necessary for discussion and are to be treated more fully elsewhere.

## III. FIELD AND EXPERIMENTAL STUDIES

In a survey of larval trematodes developing in marine mollusks of Puerto Rico, it was found that almost every individual of the snail, *Cerithidea costata*, abundant on a mud flat at Cabo Rojo, yielded at least a few tailless, unencysted distomes which were at first thought to be cercariae. After developmental stages could not be found in the snails, it was apparent that the distomes were metacercariae. Because Rees (1939) had described for a similar form a cercariaeum which developed in cockles, attention was directed to bivalves in the vicinity of snails harboring metacercariae. Among the clams present was a minute, white species, *Gemma purpurea*, in which the extremely small, furcocercous larva shown in Fig. 8 was

found to develop. That this cercaria was the one sought was established by exposing snails to infection and examining them at intervals thereafter. Next, two baby chicks were fed metacercariae and sacrificed five days later. Five mature adult trematodes were recovered from one chick but none was found in the other; worms could easily have been overlooked because of their extremely small size.

Although birds including sandpipers, plovers, terns, and herons were collected in the vicinity, none was found to harbor gymnophallines of any sort. The natural definitive host of the adults obtained experimentally may well be one of the ducks which are abundant during the winter months. Attention was not directed to this problem, however, before they had migrated.

#### IV. DESCRIPTION OF LIFE HISTORY STAGES

(All measurements are in millimeters)

Adult (Fig. 2)

##### *Parvatrema borinquenae* gen. et sp. nov.

*Specific diagnosis:* Minute distome with thick, pyriform body broadly rounded anteriorly, more pointed posteriorly; entire body with spines in quincunxial arrangement. Body length 0.19–0.195; maximum width 0.114–0.129, well anterior to ventral sucker. Oral sucker 0.05–0.06 long, 0.066–0.088 wide and not at extreme anterior end of body; with a pair of lateral papillae noticeable only in living specimens. Ventral sucker 0.022–0.025 long and 0.026–0.030 wide; its anterior margin 0.09–0.11 from anterior end of body. Prepharynx absent, pharynx 0.015 long and 0.022 wide, esophagus about length of pharynx; ceca short and widespread with thick, sparsely nucleated walls and terminating well anterior to ventral sucker. Testes opposite, slightly posterior to ventral sucker, 0.033–0.043 long and 0.02–0.03 wide; seminal vesicle apparently lacking a constriction although the prominent prostatic cells may obscure a secondary enlargement; genital pore midway between suckers, wide and pit-like. Ovary 0.038–0.048 long and 0.021–0.028 wide, anterior to right testis in adult specimens at hand. Vitellaria poorly developed, follicles in a single mass without evident division into right and left groups; evidently postero-dorsal to ventral sucker but may be displaced to left or right. Uterus extensive, with loops filling most of hind-body and extending anterior to testes on both sides, farther on the left. Eggs extremely thin-shelled and delicate, evidently about 0.014 long. Flame cell formula probably same as in metacercaria, i.e.,  $2[(2+2) + (2)]$ .

*Host:* *Gallus domesticus* (experimental); natural host probably a duck.

*Type material:* Holotype no. 47875, Helminthological Collection, U. S. National Museum.

Diagnosis of *Parvatrema* gen. nov. is given in Part VI.

The metacercaria (Fig. 1).

*Diagnosis:* Minute, unencysted distomes with many adult characters evident, including the uterus although without eggs. Heat killed specimens measure 0.198–0.238 long and 0.13–0.15 in maximum width; oral sucker 0.060–0.063 long and 0.07 wide exclusive of lateral papillae. Pharynx 0.021 long and 0.025 wide; ventral sucker 0.030 long and 0.032 wide. Testes 0.027–0.035 long and 0.024–0.026 wide; ovary 0.022–0.024 long and 0.017 wide. Spination and digestive system as in adult; ceca in life appear to be filled with granules and clear spherules which may be nuclei of the wall. Excretory pore slightly ventral at posterior end of body; vesicle prominent, filled with concretions, Y-shaped with a very short stem and long arms reaching oral sucker; each arm with three short diverticula, one partly embracing ventral sucker and a median and lateral branch at anterior end. From each median branch a ciliated excretory tubule extends posteriorly and laterally, receiving an anterior and a posterior collecting tubule at about level of genital pore. Each anterior tubule receives the capillaries of two pairs of flame cells and the posterior one pair so that the excretory formula is  $2[(2+2) + (2)] = 12$  flame cells.

*Host:* *Cerithidea costata*.

*Locality:* Sucia Bay, Cabo Rojo, Puerto Rico.

Except in size of gonads, there was little if any growth of the worms in the experimental definitive host. Actually some dimensions of the metacercaria slightly exceed those of the adult but this may be explained by differences in preparation of specimens for measurement. It therefore seems likely that the flame cell formula of the adult is the same as in the metacercaria. Rees (1939) has reported a formula

of  $2[(2+2) + (2+2)]$  for the metacercaria of a species of *Gymnophallus* whereas Prof. S. H. Hopkins has informed me in a personal communication that he has found on the Gulf Coast a gymnophalline cercaria and metacercaria with the same excretory formula that I have observed in *P. borinquenae*.

The metacercaria evidently is not deeply embedded in the host's tissues for it emerges and moves about actively when the snail is cracked but not torn apart. The distome is covered with a rather thick layer of transparent, viscous substance which remains about the worm and changes shape with its movements. I observed no calcareous masses or enclosure within cellular structures of host origin such as those reported by investigators concerned with the role of gymnophalline larvae in pearl formation. However, most such larvae occur in lamellibranchs, the metacercaria of *P. borinquenae* and "*Cercaria*" *glandosa* Lebour being exceptions found in gastropods.

The cercaria (Fig. 8)

*Diagnosis:* Minute furcocercous larva with poorly developed tail. Entire body and tail distinctly spinose but furcae without setae. Cuticle of body with a few papillae each set with a delicate bristle. Body 0.095–0.098 long and 0.04–0.042 in maximum width; tail stem 0.038–0.041 long measured from attachment to notch at base of furcae and 0.012 in maximum width at slight swelling just posterior to attachment. Furcae about 0.032 long. Oral sucker 0.02 long, 0.022–0.024 wide, and not so deeply embedded in the anterior end as in the metacercaria. Ventral sucker 0.021–0.023 in diameter and its anterior margin 0.054–0.058 from anterior end of body. A very short prepharynx is evident; pharynx about 0.012 in diameter; esophagus 0.018 long; ceca short and thick-walled, barely reaching ventral sucker. A single pair of cephalic glands occupy triangle formed by ceca and ventral sucker, overlapping these slightly; their ducts open on the oral sucker through refractile, expanded terminations remindful of the hollow cephalic spines of schistosome cercariae. Excretory vesicle filled with globules, more U-shaped than in the metacercaria and with arms not extending as far anteriorly or with as distinct diverticula. Island of Cort absent, caudal tubule prominent, constricted at intervals and with pores at tips of furcae. Tail without flame cells; two pairs observed in body. Sporocyst rather short and thick, with terminal birth pore, and containing many fully developed cercariae; difficult to separate from host's tissues.

*Host:* *Gemma purpurca*.

*Locality:* same as for metacercaria.

This diminutive cercaria is a poor swimmer. It moves body first with tail flexed ventrally at the base and lashing feebly from side to side. It belongs to the *Dichotoma* group of furcocercous cercariae distinguished by the shape of the excretory vesicle and intestine, absence of flame cells in the tail stem, and development in marine lamellibranchs. Other cercariae of this type are *C. dichotoma* Mueller, *C. fissicauda* Villot *nec* La Valette, *C. syndosmyae* Pelseneer, *C. discursata* Sinitsin, *C. baltica* Markowski, and *C. myae* Uzmamn. As in other groups of trematodes, caudal rudimentation and loss has occurred in gymnophalline larvae. Thus, although *C. discursata* has a tail, it is quickly lost and others evidently never have one and may or may not leave the sporocysts in which they develop. This may be misleading to one working with life cycles; Markowski's (1936) *Metacercaria morula* could well be a cercaria and Lebour's (1912) inability to find sporocysts or rediae of some of the "cercariae" she described was because she was dealing with unencysted gymnophalline metacercariae. It is interesting to observe how near Markowski may have come to solving a gymnophalline life history; the present study indicates that his *Cercaria baltica*, developing in *Macoma balthica* could very well be the larva of *Metacercaria mutabilis* which he reported from the same bivalve.

## V. DISCUSSION

Since it is evident that cercariae of the *Dichotoma* group are larvae of the GYMNOBALLINAE, a relationship of that subfamily to the BRACHYLAEMIDAE was suggested by Uzmann (1952) in referring *Cercaria myae* tentatively to that family. There is indeed good reason to regard such a relationship as closer than that implied by any disposition of the GYMNOBALLINAE yet proposed. When one discounts the tendency toward caudal rudimentation and loss, evidently more pronounced in the BRACHYLAEMIDAE than in the GYMNOBALLINAE, and compares the life cycle reported here with that of *Leucochloridiomorpha constantiae* as demonstrated by Allison (1943), the two have a great deal in common. The resemblance between their cercariae goes much beyond the fact that both are furcocercous with short tails. Furthermore, both larvae penetrate mollusks and become unencysted metacercariae far advanced toward the adult stage for which birds serve as definitive hosts. These, taken with many points of resemblance in their adult stages, are all evidence of a rather close relationship between the BRACHYLAEMIDAE and GYMNOBALLINAE. However, there are differences in larval and adult structure as well as host relationship that indicate a more distant affinity than a familial one. The cercaria of *L. constantiae* develops in branching sporocysts in gastropods, has penetration glands in both the oral sucker and forebody, and possesses a small spherical excretory vesicle with an Island of Cort and a pair of tubular main excretory canals; gymno-balline cercariae differ from *L. constantiae* in all of these respects.

When adults of the GYMNOBALLINAE and BRACHYLAEMIDAE are compared (cf. Figs. 3 and 4) it is seen that they have in common similar digestive systems, sparseness of vitelline follicles, relative placement of the gonads, egg size, and extent of the uterus, although with the posterior location of the genital complex, uterine coils are confined to the pretesticular region in *Leucochloridiomorpha*. Probably the most important difference is in the excretory system, especially the presence in *Leucochloridiomorpha* of a reserve network which is not shown in Fig. 4. Of less significance perhaps are differences in genitalia, position of the genital pore, and extent of the uterus. However, when taken collectively, these differences argue against assigning the GYMNOBALLINAE to the BRACHYLAEMIDAE.

Figures 6 and 7 show two other cercariae found to develop in marine bivalves in Puerto Rico. Fig. 6 is a trichocercous form so similar to the larva Palombi (1934) described for *Bacciger bacciger* that there seems little doubt that its adult is a fellodistomatid. The other is trichofurcocercous, combining caudal characters of gymno-balline and fellodistomatid larvae. To my knowledge, the only described species of its type is *Cercaria trichofurcata* which Johnston and Angel (1940) found to develop in an Australian bivalve and believed to be the larva of a species of *Tandanicola* from the swimbladder of a freshwater catfish. It seems probable that the few delicate setae restricted to the furcae of *Cercaria myae* and *C. discursata* and perhaps those generally distributed over the tail of the larva of *Leucochloridiomorpha* correspond to those so much better developed in the two trichofurcocercous species. A striking difference between all of these and the furcocercous larvae of most strigeoids, blood flukes and their relatives is the absence of flame cells in the tail although the caudal excretory tubule is extremely prominent. This lack of flame cells might be explained by the poor extent to which the tail is developed in the GYMNOBALLINAE and *Leucochloridiomorpha* were it not for their absence also

in the species shown in Fig. 7 in which the tail is an extremely well developed and efficient swimming organ. Hence, this difference in caudal structure of various furcocercous species is believed to be a fundamental one.

There is good evidence that the cercariae shown in Figs. 6–8 may be closely related. This is not so much from the trichocercous-trichofurcocercous-furcocercous intergrading series in which they can be arranged, as from the structure of their bodies, especially the excretory system. All three have voluminous, thin-walled excretory vesicles filled with concretions and with arms extending well into the fore-body. From each arm, a ciliated recurrent tubule extends backward and receives a pair of collecting tubules. While the excretory pattern of the trichofurcocercous species is complex, the formula for its sporocyst (Fig. 7a) is identical to the formula of the trichocercous larva itself (Fig. 6) and is empirically the same as in the gymnophalline metacercaria (Fig. 1). Further support of the view that these cercariae are closely related is the fact that all of them develop in bivalve mollusks. Host relationships of larval trematodes have been rather generally ignored in taxonomic concepts, more emphasis having been given to adult stages and their hosts which as growing evidence indicates, are often a matter of opportunism so far as the digenetic trematodes are concerned.

Indication that the GYMNOHALLINAE and FELLODISTOMATIDAE are related is by no means restricted to larval stages. In fact, when their adults are compared, it is surprising that an affinity between them has not been proposed. Neither the fact that the GYMNOHALLINAE are parasites of birds whereas the FELLODISTOMATIDAE occur in fishes nor the absence of a cirrus sac in one and its presence in the other is sufficient evidence of a family distinction. Actually, Yamaguti (1938) was unable to find a definite cirrus sac in *Bacciger harengulae* and allocated the species to the family HETEROPHYIDAE. Later, Manter (1947) likewise was unable to find that structure in *B. harengulae* but assigned to the FELLODISTOMATIDAE this form which in all respects except the matter of a cirrus sac is typical of that family.

Figs. 3 and 5 are respectively the type species of *Gymnophallus* and a fellodistomatid found repeatedly in the intestine of the black angel fish in Puerto Rico. The major differences between these trematodes is the absence of a cirrus sac in *Gymnophallus*, position of the vitelline follicles, and location of the ovary. Other fellodistomatids could be selected which are even more like *Gymnophallus* in these respects; *Bacciger harengulae* has already been cited as one evidently lacking a cirrus sac. A bipartite seminal vesicle occurs in both groups and of particular interest is the position of the genital pore in Figs. 3 and 5. In the fellodistomatid, it is actually within the rim of the ventral sucker and it is either at the edge of the sucker opening or possibly within the sucker cavity in *Gymnophallus*. There is further agreement not only in the shape and extent of the excretory vesicle but in the flame cell pattern as well.

From the foregoing discussion, it seems evident that the GYMNOHALLINAE are more closely related to the FELLODISTOMATIDAE than to any other family of digenetic trematodes and that differences between them are not of sufficient magnitude to justify the erection of a distinct family for the GYMNOHALLINAE. It is therefore proposed that the FELLODISTOMATIDAE be emended to receive the GYMNOHALLINAE as a subfamily.

Johnston (1927) erected for *Tandanicola bancrofti* the subfamily TANDANICO-

LINAE which he placed in the BRACHYCOELIIDAE. According to his description, the genus *Tandanicola* differs in but one respect from accepted genera of the FELLODISTOMATIDAE, viz., complex development of the genital atrium as a copulatory structure in the absence of a cirrus sac. The fellodistomatid genera *Pseudosteringophorus* Yamaguti and *Megalomyzon* Manter also show considerable modification of the genital atrium although a cirrus sac is present. A careful comparison of the so-called copulatory sac of *Tandanicola* with the atrial sac of *Megalomyzon* and *Pseudosteringophorus* suggests that these three genera comprise a related group showing progressive development of accessory structures with the loss of the more primitive cirrus sac. Although such modifications are well known in other families, there they are of fundamentally dissimilar types. It is proposed, therefore, that the subfamily TANDANICOLINAE be redefined to include besides the type genus also *Megalomyzon* and *Pseudosteringophorus* and that the subfamily be transferred to the FELLODISTOMATIDAE. Johnston and Angel (1940) suggested that *Cercaria trichofurcata* might be the larva of *Tandanicola bancrofti*, which if true would support the proposed disposition of the TANDANICOLINAE. It is possible that the adult of the trichofurcocercous larva shown in Fig. 7 also may belong to this group although the cercaria resembles adults of another subfamily, the HAPLOCLADINAE, in several respects. In *Tergestia*, however, the only haplocladine genus for which the excretory pattern is known, the flame cell number is much smaller than in the cercaria shown in Fig. 7, thus indicating that the adult cannot be a species of that genus at least.\*

A relationship of the GYMNOBALLINAE and hence the FELLODISTOMATIDAE to the BRACHYLAEMIDAE is implied by earlier discussion. The chief differences in their adults are the presence of a reserve excretory network and the posterior position of the genital pore in the BRACHYLAEMIDAE. In several fellodistomatids, Laurer's canal is very long and extends almost to the posterior end of the body before opening to the dorsal surface. This may indicate a migration of the genital complex and with it the genital pore from a more primitive posterior location. Certainly the position of the genital pore in the species shown in Fig. 5 is an exceptional one. This form has another interesting and puzzling feature, viz., a well developed pit on the dorsal surface near the bifurcation of the excretory vesicle. It is not associated with the opening of Laurer's canal. An anterior migration of the genital pore evidently has occurred in the Superfamily SCHISTOSOMATOIDEA and within a single family the SPIRORCHIDAE, its position varies from near the posterior end of the body to the level of the ventral sucker.

A taxonomic scheme designed to show relationships between families should

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\* ADDENDUM

Two papers that appeared too late to be included above should be mentioned. In one, Hutton (1952 J. Marine Biol. Assoc. United King. 31: 317-326) described *Cercaria fulbrighti*, a furcocercous gymnophalline larva from *Cardium edule*. In that cercaria, the tail forms and degenerates within the sporocyst and what is described as "fully developed cercariae" may correspond to the metacercaria of *P. borinquense*. Hutton believes that the adult is a species of *Gymnophallus* and suggests a relationship of the Gymnophallinae to the Strigeatoidea as postulated above. In the second paper, Dubois, Baer and Euzet (1952 Rev. Suisse Zool. 59: 503-510) described from marine plankton *Cercaria mathiasi*, a furcocercous species that can hardly be other than the larva of *Tergestia*. Their observations lend significant support to the writer's views concerning relationships which in turn suggest that the molluscan host of *C. mathiasi* will be a lamellibranch when found.

express the affinity that seems to exist between the BRACHYLAEMIDAE and FELLODISTOMATIDAE. Toward that end, it is proposed that the superfamily BRACHYLAEMOIDEA Allison be emended to receive these groups. Allison (1943) observed that certain characteristics of the BRACHYLAEMIDAE and BUCEPHALIDAE set them apart from other families of the STRIGEATOIDEA. Unfortunately, miracidial structure is unknown in the FELLODISTOMATIDAE; belated attempts to observe development within the minute eggs of some of these trematodes were unsuccessful. However, Allison's observation is supported by the present study to the degree that it might be suggested that the BUCEPHALIDAE, BRACHYLAEMIDAE and FELLODISTOMATIDAE represent a distinct phylogenetic group within the STRIGEATOIDEA. To such a concept, the gymnophalline cercariae contribute the interesting aspect of being fundamentally similar to brachylaemid larvae, yet developing in bivalve mollusks as do cercariae of the BUCEPHALIDAE.

Recent studies have extended La Rue's (1926) concept dividing the DIGenea into two great groups, the STRIGEATOIDEA and PROSOSTOMATA. Yet their members have so much in common that it seems certain that they have descended from a common ancestral stock considerably removed from the turbellarians which are generally believed to have been their ultimate source. It is likely that further knowledge will reveal that certain families are close to the point of divergence of the STRIGEATOIDEA from the PROSOSTOMATA. Whether the FELLODISTOMATIDAE with such larval types as those shown in Figs. 6-8 may be such a family is an interesting speculation but no more than that at the present.

#### VI. TAXONOMY

Desirable as it would be, a taxonomic scheme serving as an easily used key to the higher categories as interpreted in this study is not feasible. Differences in adult structure preclude concise and unqualified characterizations of suprageneric groups. Although some of this variation is correlated with differences in body shape, more is attributable to divergence within a genetically related group.

##### Superfamily BRACHYLAEMOIDEA Allison, 1943, *emend.*

STRIGEATOIDEA. Distomes of various shape. Prepharynx very short or absent, pharynx present but sometimes poorly developed, esophagus present or absent and elongate only in some slender forms. Intestine triclad or rarely with a single cecum; ceca variable in length. Excretory system either with a small bladder with tubular canals extending into forebody, or these greatly expanded, the whole forming a voluminous V-, U- or Y-shaped vesicle with arms reaching well anterior to ventral sucker except in a few elongate forms; recurrent tubule often ciliated, short or reaching almost to posterior end of body. Flame cells commonly in four groups on each side of body. Genital pore variable in position, atrium rarely modified, cirrus sac present or absent. Arrangement of gonads variable, Laurer's canal present and sometimes enlarged to form a seminal receptacle. Eggs usually medium to small in size and numerous. Sporocysts simple or branched, in gastropods or lamelli-branchs. Cercariae furcocercous, trichofurcocercous, trichocercous, microcercous or tail-less; tail without flame cells but with prominent excretory tube and pores when forked or even reduced to a mere knob. Metacercariae in invertebrates, often as unencysted distomes in mollusks. Definitive hosts all vertebrate groups except

cyclostomes and elasmobranchs. Includes the families Brachylaemidae and Fellodistomatidae. See Allison (1943) for an emended diagnosis of the Brachylaemidae.

Family FELLODISTOMATIDAE Nicoll, 1935, *emend.*

(The name of this family has been attributed to Odhner (1911) although he called it STERINGOPHORIDAE, deliberately renaming the older subfamily FELLODISTOMINAE Nicoll as STERINGOPHORINAE with the excuse that the genus *Fellodistomum* did not seem typical of the group. Odhner was not consistent for he went on to select as type of another subfamily the genus *Haplocladus* which with its single cecum is perhaps the least typical one of the entire family. Some authors have recognized the name FELLODISTOMIDAE; that used here is the slightly different form given by Nicoll in the Zoological Record for 1935.) BRACHYLAEMOIDEA. Rather small to minute distomes with thick bodies which may or may not be spinose. Ceca short to moderately long; intestine rarely with a single cecum. Genital pore median or somewhat lateral, anterior to ventral sucker, on its anterior lip, or rarely within its cavity. Usually, prostatic cells well developed and seminal vesicle constricted; spermatophore formation described for some species. Genital complex usually well removed from posterior end of body. In less elongate forms, gonads in triangular arrangement either with ovary anterior to one testis or more median and ranging from a level anterior to one somewhat posterior to testes; in elongate forms, gonads more or less in tandem with ovary anterior-most. Vitelline follicles scanty as a rule, either anterior or posterior to ventral sucker, rarely both, and never reaching posterior end of body. Seminal receptacle small or absent, uterus with loops posterior to gonads. Excretory bladder voluminous, with arms extending to pharyngeal level except in some elongate forms. Excretory formula of adult where known is  $2[(n+n) + (n+n)]$  or  $2[(n+n) + (n)]$ ; often  $n = 2$  or  $3$ . Cercariae develop in lamellibranchs; metacercariae in crustaceans or mollusks. Adults in intestine and gall bladder of water birds and marine fishes, rarely in swim bladder of a freshwater fish.

Subfamily FELLODISTOMATINAE Nicoll, 1909, *emend.*

FELLODISTOMATIDAE. Body not elongate with gonads in tandem or nearly so. Genital pore median or lateral, anterior to ventral sucker or rarely within its anterior lip. Esophagus short or absent, ceca short to moderate in length. Genital atrium simple, cirrus sac rarely not evident. Vitelline follicles lateral, usually anterior and rarely both anterior and posterior to ventral sucker. In the one life history reported, the cercaria is non-ocellate and trichocercous with setae in finlet-like paired groups, excretory vesicle U-shaped; an amphipod serves as second intermediate host. Parasites of marine fishes. Several genera.

Subfamily HAPLOCLADINAE Odhner, 1911, *emend.*

Fellodistomatidae. Body elongate, sometimes with anterior circle of papillae. Intestine a single cecum or paired and then either terminating anterior to ventral sucker or with a long esophagus so that ceca are confined mostly to hind-body. Genital pore anterior to ventral sucker, usually not median; cirrus sac present, genital atrium not modified, gonads in tandem. Vitellaria lateral, posterior to ventral sucker. Life history unknown; cercariae possibly furcocercous (see Odhner,

1911) or trichofurcocercous. Includes the genera *Haplocladus* Odhner, *Tergestia* Stossich, *Proctoeces* Odhner and *Ancyclocoelium* Nicoll.

Subfamily TANDANICOLINAE Johnston, 1927, *emend.*

Fellodistomatidae. Body short and thick to moderately elongate, ceca not extending much posterior to ventral sucker. Genital atrium modified to form an atrial sac when a cirrus sac is present or a complex structure with a copulatory papilla in the absence of a cirrus sac. Vitellaria lateral, at or anterior to level of ventral sucker; gonads in triangular arrangement, near or even anterior to ventral sucker. Life history unknown; cercaria possibly trichofurcocercous (see Johnston and Angel, 1940). Adults in intestine of marine fishes and swim bladder of a freshwater siluroid. Includes the genera *Tandanicola* Johnston, *Pseudosteringophorus* Yamaguti and *Megalomyzon* Manter.

Subfamily GYMNOPHALLINAE Odhner, 1905, *emend.*

FELLODISTOMATIDAE. Small to minute distomes with oval or pyriform, spinose bodies. Ceca short, never reaching much posterior to ventral sucker. Genital pore median, either some distance from ventral sucker, on its anterior lip, or possibly within its cavity. Genital atrium simple, cirrus sac absent, gonads in triangular arrangement. Vitelline follicles few, compact and near median line close to ventral sucker. Arms of excretory vesicle may have short diverticula. Cercariae furcocercous or tail-less, metacercariae unencysted in mollusks although often embedded in a mucoid substance or enclosed in capsules of host origin and stimulating pearl formation. Adults in intestine and gall bladder of aquatic birds. Includes the genera *Gymnophallus*, *Gymnophalloides* and *Parvatrema*.

*Gymnophallus* Odhner, 1900, *emend.*

GYMNOPHALLINAE with terminal genitalia arching ventrally and then posteriorly; genital atrium narrow and elongate, pore inconspicuous and on anterior lip of ventral sucker or possibly within it. Pharynx usually small. Vitellaria recognizable as two masses of follicles. Excretory formula of metacercaria and probably adult  $2[(2+2) + (2+2)]$ . Cercaria where determined is tail-less but probably is furcocercous in some species. Type species, *G. deliciosus* (Olsson) Odhner.

*Parvatrema* gen. nov.

Similar to *Gymnophallus* except that the genital pore is large, pit-like and distinctly anterior to ventral sucker, the genital atrium is short and does not extend posteriorly, the pharynx is well developed, in the one known adult two groups of vitelline follicles are not recognizable, and the metacercarial excretory formula is  $2[(2+2) + (2)]$ . Its cercaria is furcocercous and the metacercaria is in a gastropod. Type and only species, *P. borinquenae*. The structure of certain described metacercariae suggests that their adults if known would belong to the genus *Parvatrema*. In one of these (Markowski, 1936), the presence of a sucker-like structure at the genital pore indicates that its adult may represent still another genus of the subfamily Gymnophallinae.

*Gymnophalloides* Fujita, 1925

This genus is known only from the metacercaria in a Japanese oyster. Fujita's description is replete with errors of interpretation some of which Dollfus mentioned in translating the paper for publication. The figures show, however, that the form is very similar to *Parvatrema*. The resemblance is so close, in fact, that the two genera may be synonymous; with liberal reinterpretation of Fujita's description, the principle differences between them would seem to be the form of the vitellaria and the class of mollusks serving as the second intermediate host. Because the adult of *Gymnophalloides* is unknown and nothing is given concerning the excretory pattern, that genus and *Parvatrema* are considered distinct for the time being.

## VII. SUMMARY

The life cycle of *Parvatrema borinquenae* gen. et. sp. nov. was traced experimentally. The cercaria is a minute furcocercous larva developing in a marine clam, *Gemma purpurea*, and becoming an unencysted metacercaria in *Cerithidea costata*, a gastropod. Mature adults were obtained in chicks. The GYMNOPHALLINAE are placed in the family FELLODISTOMATIDAE which is emended to receive also the subfamily TANDANICOLINAE to which is assigned the genera *Pseudosteringophorus* and *Megalomyzon*. The family FELLODISTOMATIDAE is assigned to the superfamily BRACHYLAEMOIDEA to express affinity with the BRACHYLAEMIDAE.

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## EXPLANATION OF FIGURES

## PLATE I

- FIG. 1. Metacercaria of *Parvatrema borinquense*.
- FIG. 2. Adult of *P. borinquense*.
- FIG. 3. *Gymnophallus deliciosus*, redrawn from Odhner (1900).
- FIG. 4. *Leucochloridiomorpha constantiae*, redrawn from Allison (1943) with part of excretory system added.
- FIG. 5. A fellodistomatid from the black angelfish. dp, dorsal pit; lc, Laurer's canal.

## EXPLANATION OF FIGURES

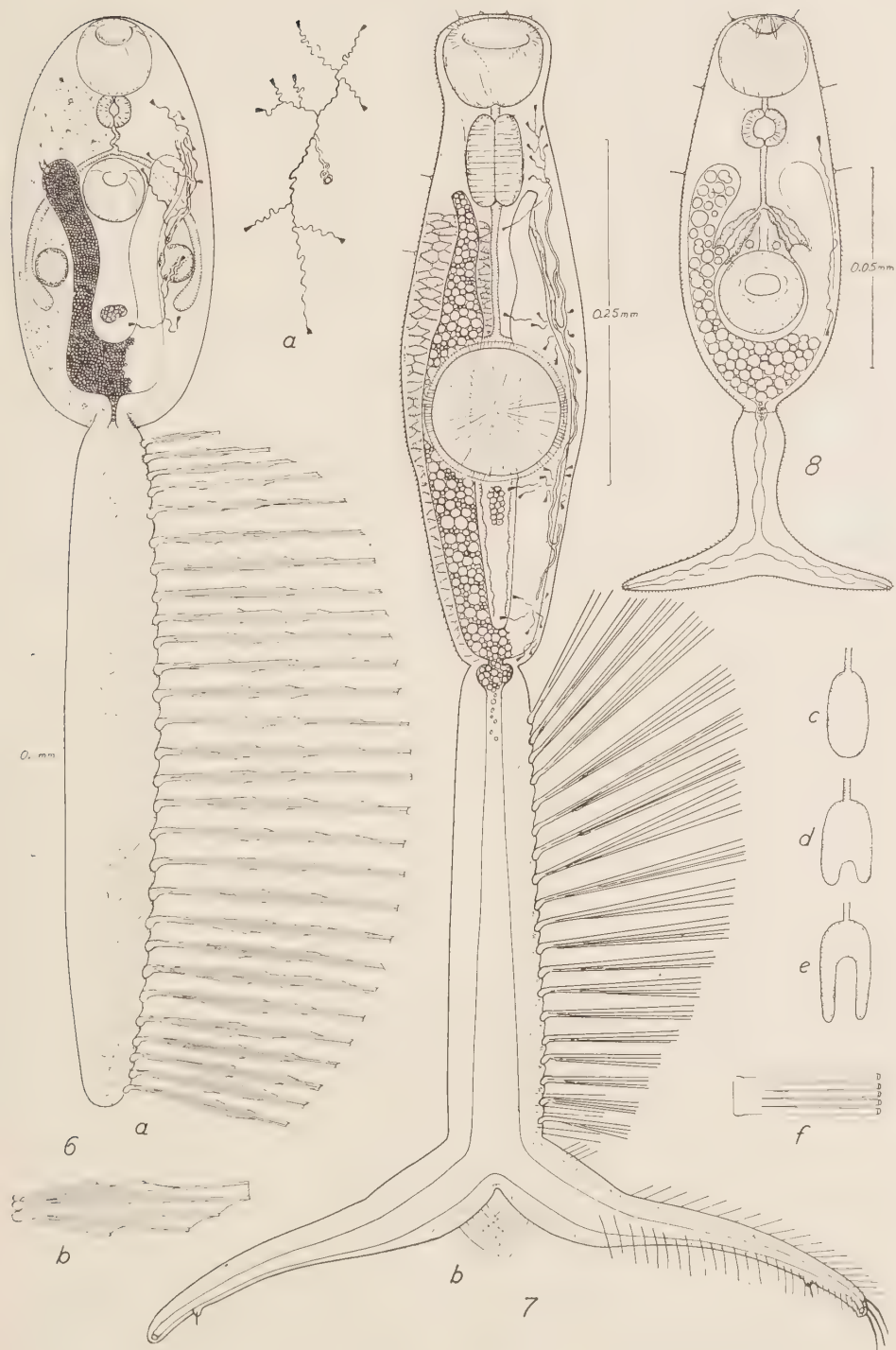
## PLATE II

- FIG. 6. Trichocercous cercaria. a, ventral view with caudal appendages omitted from one side; b, detail of caudal appendage.
- FIG. 7. Trichofurcocercous cercaria. a, excretory system of one side of sporocyst; b, cercaria in ventral view with details of caudal structure omitted from one side; c, d and e, development of triclad from rhabdocoele intestine in cercarial embryo; f, basal portion of setaceous tuft on tail-stem.
- FIG. 8. Cercaria of *Parvatrema borinquense*.

PLATE I



## PLATE II



# AXENIC CULTIVATION OF THE PARASITIC NEMATODE, *NEOAPLECTANA GLASERI*, IN A FLUID MEDIUM CONTAINING RAW LIVER EXTRACT

NORMAN R. STOLL

The Rockefeller Institute for Medical Research, New York 21, N. Y.

*Neaplectana glaseri* Steiner, 1929 originally discovered in grubs of the Japanese beetle, *Popillia japonica* Newm. by Glaser and Fox (1930), is now known to parasitize several species of insects, especially coleopterous larvae inhabiting the soil (Christie, 1941; Swain, 1943; Dumbleton, 1945). In a series of experiments which demonstrated the value of the nematode as a biological control agent against the Japanese beetle, Glaser (1931, 1932, 1940a), McCoy and Glaser (1936), and Girth, McCoy and Glaser (1940) worked out methods of growing the oxyurid non-sterilely on veal infusion agar and on ground raw potatoes seeded with live yeast, and on veal pulp (McCoy and Girth, 1938). With a technique devised by Glaser and Stoll (1940) for sterilizing *Haemonchus contortus* larvae, Glaser (1940b) later reported success in ridding *N. glaseri* of contaminants and culturing it bacteria-free. Successive life cycles would ensue on pieces of fresh, sterile rabbit kidney on dextrose agar slants, and the organism could be serially transplanted, apparently indefinitely. This exceptional result of generation following generation axenically *in vitro*, without return to the host, was the first recorded for a worm parasite. It has been reported for only one other species, the closely related *Neaplectana chresima*, by Glaser, McCoy and Girth (1942), who were able to carry it sterilely for 2 years through 28 transplants, although it failed to survive when bacterially contaminated. Culture "in liquid media containing kidney extracts devoid of particulate matter" was also briefly noted for *N. glaseri* (Glaser, 1940b), but without details.

Several months after Dr. Glaser's death, the present author, impressed by the potential value for experimentation of a parasitic nematode that could be carried in the laboratory on stock cultures, began a study of it in fluid media. In contrast to the ease of culturing the organisms on kidney tissue, growth and cyclical development in kidney extracts or in other liquid media was not initially obtained. This difficulty was later resolved by adding to veal infusion broth an aqueous extract of raw liver (RLE), prepared without heat, acidified, and sterilized by filtration (Seitz). The present report deals with its use, and concomitant conditions found favorable for culturing. Results obtained at the Princeton laboratory prior to July 1950, have been confirmed and extended at New York.<sup>1</sup>

## I. LIFE HISTORY OF *N. glaseri* AND USE OF STOCK CULTURES

1. *Life history.* As a parasite, *Neoplectana* has both common and unique nematode characteristics (Glaser, 1932). Common is the fact that it passes through four larval stages—each ending in ecdysis—and an adult stage, with the 3rd stage infective larvae surviving in nature between hosts, as is true of hookworm and *Haemonchus*. Unique is the characteristic that the usual sequence of a nematode para-

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<sup>1</sup> Acknowledgement is made of the technical assistance of Mary Ann Getchell (now Mrs. T. J. Lowery) and Patricia J. White.

site developing a fraction of its life cycle within one host and then being under the necessity of leaving that host for the life cycle to be continued, has here added to it an ability to undergo at times more than one complete life cycle within one host. Development occurs in living grubs infected with *N. glaseri*, and probably with *N. chresima* (Glaser, McCoy and Girth, 1942), but in the related species *N. affinis* and *N. bibionis*, Bovien (1937) found development and multiplication do not take place until their insect hosts die.

Under natural conditions *N. glaseri* is believed to be passively ingested in the 3rd larval stage in soil, although active invasion of the grub through mouth or anus may also be a method of entrance. In the gut of the grub the nematodes complete the 3rd and 4th larval and bi-sexual adult stages, and the fecundated ovoviviparous females begin to bear young 2 to 4 days after parasitization. Under favorable conditions these new 1st stage larvae grow up and in turn continue the cycle, a complete generation requiring less than a week. When worms are numerous they may invade the entire body of the grub, even head and legs, and kill the host. Life cycle conditions become unfavorable after death of the grub, and the nematodes die except for such as are able to reach the 3rd stage. Survivors persist in moist soil for many months. Examined under the microscope with transmitted light, they have the black, well-fed appearance of infective 3rd stage larvae of hookworm or *Haemonchus*, and have been called "dauer larvae" by Bovien (1937), a term earlier introduced by Fuchs (1915). If they dry, they die.

2. *Stock cultures.* In axenic stock cultures on kidney tissue on dextrose agar slants as devised by Glaser (1940b), the characteristic cycle noted above also takes place. After two or three generations in a period of 2–3 weeks, the kidney habitat becomes unfavorable. Larvae then tend to leave the culture mass and migrate to the glass walls of the tubes (Fig. 1), an activity suggestive of their migration into soil from grub cadavers. The tubes may be refrigerated at this time without apparent damage to the migrants, unless they dry.

Migrants that have wandered farthest from the surface of the medium are found on microscopic examination to be well-fed 2nd and 3rd stages, readily distinguishable by whether or not they have undergone ecdysis at the end of the 2nd stage. They are recoverable in large numbers from the walls of the tube with the aid of a heavy bacteriological loop.

Instead of re-culturing them at once on kidney or in fluid media, they may be transferred for storage to sterile distilled or well-water a few mm. deep in cotton-plugged, small Erlenmeyer flasks to the number of several tens of thousands of worms in 10–20 ml. water. In the refrigerator at 2 to 5° C., they retain viability (Fig. 2) and sterility for months (*i.e.*, stock harvested 18 December had 93% of the larvae active on 18 May, when used for sub-culture), but they die if frozen.

Larvae not removed from the walls of the tube after their migration from culture, later desert the glass and bury themselves in the agar itself. They can be caused to re-migrate out of the agar in considerable numbers by flooding it with water.

Stock cultures are made in test tubes 22 × 180 mm., with slants of 10 ml. of 2% infusion agar containing 1% dextrose. At the bottom of the slant is placed a piece of fresh sterile kidney weighing about 1 gm., *i.e.*, approximately  $\frac{1}{8}$  portion of adult rabbit kidney. This is inoculated with an aqueous suspension of 100–200 infective larvae, or by mixed stages from another stock or fluid culture. Sterile kidney may be kept refrigerated a few days before use, but after a period, variable for different lots, but sometimes within a week—sooner at room temperature—tends to

lose capacity to grow thriving cultures. Frozen kidney retains its value for stock cultures longer.

As substitute tissue, sterile liver from rat and guinea pig, and guinea pig testis have produced even richer cultures than kidney. Kidney from a 6-month-old bull calf was also especially favorable. Rat spleen made a poor culture medium.

After inoculation the tubes are placed nearly horizontally in a tray in the dark at room temperature. It is preferred to add water in small amounts (0.1–0.3 ml. at a time) if laboratory conditions of low humidity prevail, rather than to seal the tubes.

The axenic strain now in use was originally isolated from a Japanese beetle grub and rendered bacteria-free in Dr. Glaser's laboratory in 1944. It was maintained on

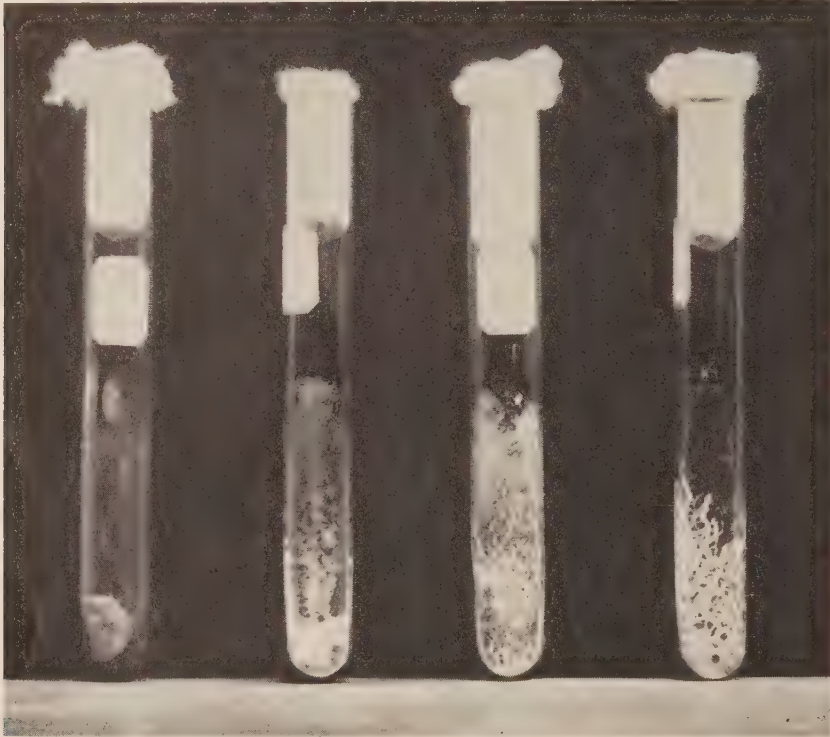


FIG. 1. Stock cultures of *N. glaseri* on sterile rabbit kidney on dextrose agar slants. Left to right: uninoculated kidney; culture 11 days after inoculation, worms mostly confined to tissue; on 20th day, after preliminary harvesting of larvae 2 days before, and with culture forms distributed over surface of slant; thread-like matting of larvae after migration to wall of culture tube, at 3 weeks incubation. Photograph by J. A. Carlile.

stock cultures serially by monthly transfer, usually on rabbit kidney, sometimes on chick embryo tissue (Glaser, 1943). About 132 generations are estimated to have elapsed when it was received in February, 1948,<sup>2</sup> after 44 months axenically *in vitro*.

It has since been carried in similar fashion serially, at first on chick embryo tissue, but mostly on rat, guinea pig and rabbit kidney (occasionally on autoclaved kidney supplemented with RLE as in V, 1), with intermediate refrigerator storage of the dauer larvae in water as noted above. In the 56 months to Oct., 1952, the strain has continued vigorous and flourishing after 23 serial transfers and an estimated 69 additional generations.

<sup>2</sup> The author is indebted to Nicholas B. Coria for this stock.

## II. PRELIMINARY EXPERIMENTS

1. *Initial attempts with fluid cultures.* The difficulty first encountered, in getting axenic 3rd-stage larvae to develop at all in fluid media, was true even in unheated extracts of kidney or liver rendered sterile by filtration (Berkefeld N or fritted glass). Controls inoculated on portions of the same tissue from which the extracts were derived gave typically good results on dextrose agar slants.

It seemed at the time that this meant an inability of the worms to grow at the bottom of even a short column of fluid. They are poor swimmers and do not by their own efforts rise vertically in liquid more than a few millimeters. Various sterilized substrates were accordingly offered, such as agar of various strengths, sand, glass beads, asbestos, charcoal, cellulose sponge, and glass wool. In tubes then placed in a slanted position these substrates protruded peninsula-like from the fluid medium, giving gradients for depth and oxygen tension. All substantially failed on

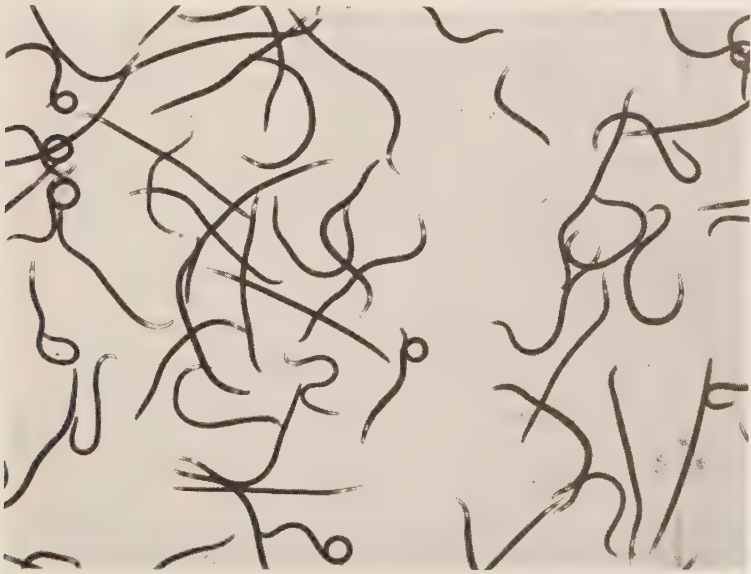


FIG. 2. Axenic 3rd stage ("dauer") larvae of *Neoplectana glaseri* harvested from wall of stock culture tube, after refrigerator storage in water 18 days. Cast 2nd stage sheaths visible. Mag.  $\times 20$ , in water, no coverslip. Photoflash (1/200 sec.) J. A. Carlile.

test, for infective larvae introduced to such cultures (including some made with synthetic media) rarely showed signs of any development whatever. So constant a finding was this that exceptional significance was early attached to the occasional presence of even a few young adults, and given undue emphasis (Stoll, 1948).

Better cultures followed the encouraging observation that in veal infusion broth tubes held at room temperature as sterility controls of larval inocula, some worms grew to the adult stage. Occasionally young were found, but only after delay beyond the normal period exhibited by stock cultures. Such offspring failed for the most part to develop properly, and reproduced little if at all.

Supplementation was then undertaken with a number of substances. Various dilutions of commercial liver extracts, both crude and purified (nine commercial brands tested), yeast extract, casein hydrolysate, casein factor<sup>3</sup> of Price (1948),

protogen,<sup>3</sup> B<sub>12</sub> (Cobione), Simms ultrafiltrate, horse and bovine serum, ascitic fluid,<sup>3</sup> and milk, when added to broth, all have failed to give development differing from that of minor degree induced by broth alone.

Not until an aqueous extract of raw liver (RLE), prepared without heat and sterilized by filtration, was added to veal infusion broth, did fluid cultures for *N. glaseri* thrive and multiplication take place freely. After a year's experimentation, worm yields at 3 weeks represented  $\times 10$  to  $\times 30$  fold the number of infective larvae introduced as inoculum, but after a second year,  $\times 100$  to  $\times 200$  fold yields were frequent and have since been considered the rule.

Inconsistencies in results, experiment to experiment, and extract to extract, were decreased following the recognition of several factors.

2. *Size of inoculum.* Introducing large and unknown numbers of larvae into cultures was a convenience, but less reliable as a test procedure than the use of small and known numbers. The likelihood existed—analogueous to the situation with certain bacteria—that substances freed from the disintegration of sterile worms when numerous in unfavorable media, would tend to favor development of those still alive, and thus mask the testing of other factors.

Supporting this possibility was one experimental observation. The worms from several successful fluid cultures were retrieved under aseptic conditions and washed repeatedly over a 3-day period with large amounts of sterile broth and physiological saline to remove traces of the media in which they had been cultivated. They were then triturated in 0.85% saline in a Ten Broeck grinder and the brei added as a supplement to veal infusion broth in a test run with 25 infective larvae. This tube in 3 weeks produced 560 worms ( $\times 22$  fold yield) of which 162 were adults, when 4 control tubes with unsupplemented broth produced an average of 81 worms ( $\times 3$  fold yield), of which 49 were adults.

While this single experiment did not furnish proof of the assumption above, it favored the idea that the inoculation desirable as a standard to test culture conditions should be with few larvae. Their number should be sufficiently low so that initially only a small number of mating pairs of adult worms would be present in the culture, yet without inviting unduly the biological hazard of lopsided sex ratios from the statistical variation inherent in excessively small samples. The male:female ratio of worms developing from fair samples of *N. glaseri* larvae appears to be 1:1. The number of infective larvae chosen as a standard inoculum was 25. Later results (IV, 7) indicated that under the conditions of the succeeding experiments this was a useful and sometimes critical number.

3. *Incubation period.* It was hoped that a brief incubation period, such as a week, might prove to be adequate for a tube of 10 ml. of liquid medium to demonstrate its capacity to foster multiplication of *N. glaseri*. However, culture yield at 1 week was not found to necessarily reflect the magnitude demonstrable later from the same tube. Some worm populations in the tubes get off to a better start than others. Also it was found that re-counting tubes at intervals, even though fluid removed for counting was restored, did not allow the development of as large yields per tube as when they were left undisturbed.

A favorable point to determine culture yields proved to be 3 weeks incubation, as indicated in Fig. 3.

<sup>3</sup> The author is indebted to Dr. W. H. Price of The Rockefeller Institute for Medical Research, and to Dr. E. L. R. Stokstad of Lederle Laboratories, respectively, for test samples of casein factor and protogen, and to Dr. R. J. Slater of the R.I.M.R. for the ascitic fluid.

In this experiment 40 tubes were similarly prepared with 9 ml. beef heart infusion supplemented with 1 ml. rabbit RLE and inoculated with 24 infective larvae. Tubes for examination at weekly intervals were chosen at random and discarded after counting. All tubes remaining were counted at 6 weeks. From the combined standpoint of mean yield and per cent of live worms present, 3 weeks incubation was optimum for examination. The decreased average yields at the end of 4, 5, and 6 weeks are ascribed in part to the disintegration of young worms unable to develop, by reason of which they were lost to the count.

4. *Acid media.* The RLE has a pH of 4.3–5.0. With it development and multiplication of the nematode was demonstrated through a wide pH range, from less than 5.0 to more than 9.0. Glaser, McCoy and Girth (1942) had recommended pH 7.0 for an autoclaved semi-solid gel medium for *N. chresima* and *N. glaseri*. How-

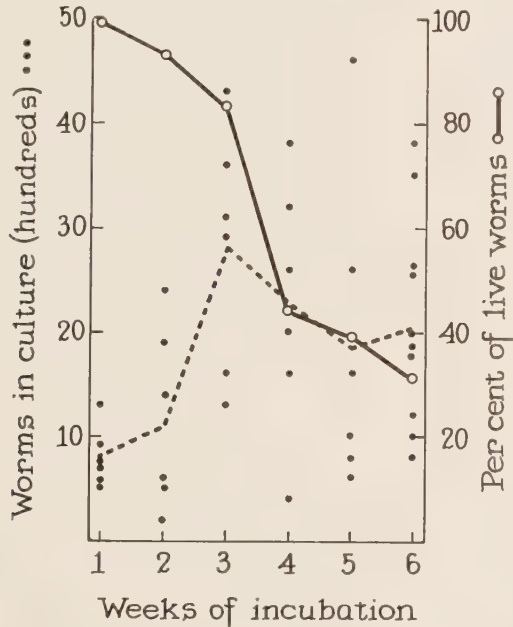


FIG. 3. Illustrating yields of *N. glaseri* in relation to weeks of incubation. Data from cultures begun at pH 6.2 with 9 ml. beef heart infusion broth supplemented with 1 ml. rabbit RLE, and inoculated with 24 infective larvae. The dotted line connects mean values from tubes selected at random for counting at weeks 1–6; full line, percentage of live worms in culture. See text.

ever, fluid cultures for *N. glaseri* begun at pH 6.0–6.5 were found to give more satisfactory yields. (See also IV, 6.)

5. *Temperature.* *N. glaseri* being a parasite of a poikilothermic host, at first no special attention was paid to temperature conditions beyond keeping cultures at "room temperature." Stock cultures were characteristically successful on the laboratory table.

A danger in being too casual about this aspect of the problem was pointed up when on March 23, 1950, the shakers at the Princeton laboratory were moved to a large darkened room. The windows had full exposure to afternoon sun but were heavily curtained. This move was followed by a 2-month period of failure to obtain accustomed development in cultures. A summary of dry-bulb readings routinely made in the rooms used for experiments, provided revealing information. Temperatures in the culture room by 10-day averages for the 50 days preceding

March 23, had been 24.7, 24.4, 25.3, 24.5, and 26.3° C., associated with good cultures. After the move, the 10-day averages was 28.6, 28.7, 28.2, 28.9, 29.9, and 31.8° C., associated with increasingly disappointing cultures.<sup>4</sup>

Temperature means of 21–26° C. (about 70–80° F.) appear to be favorable.

6. *Shaking of cultures in the dark.* Tubes maintained in a shaking machine were found to give better yields of worms. A similar observation was made concerning those kept in the dark.

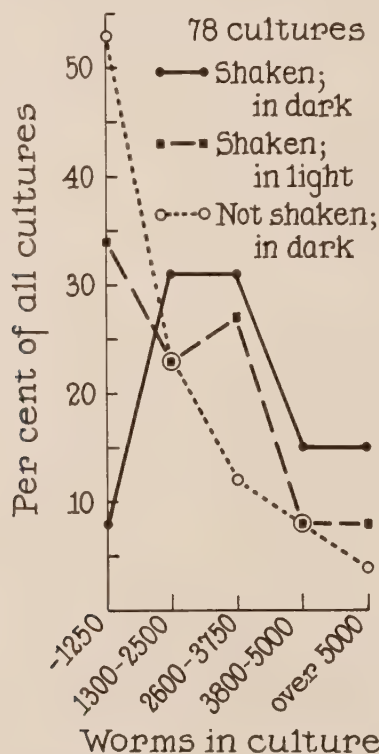


FIG. 4. Percentage distribution, in various categories of yield, of three groups of *N. glaseri* cultures inoculated with 25 larvae and counted after 3 week's incubation. See text.

A reasonably definitive measure of the influence of these factors was not made until the period Dec. 1950–March 1951, when a constant temperature room was available. Then in various experiments extra culture tubes, similarly prepared and inoculated with 25 larvae, were used to determine comparative yields between tubes shaken in the dark, tubes shaken in the light on a table top before an east window but protected from sunshine, and tubes kept in the dark room but not shaken. Tubes were maintained uninterruptedly under each set of conditions throughout the 3-week culture period.

There were 78 cultures in which the same number of tubes counted at 3 weeks from a given experiment were represented in each of the three sets of conditions. Fig. 4 is arranged to show comparatively the percentage of cultures obtained when grouped by yield, up to 1250, 1300–2500, 2600–3750, 3800–5000, and over 5000 worms. These are respectively  $\times 50$ ,  $\times 100$ ,  $\times 150$ ,  $\times 200$ , and greater than  $\times 200$  fold yield from inocula of 25 larvae. Here 26 cultures shaken in the dark averaged 3340 worms, 26 parallel cultures shaken in the light averaged 2480 worms, and 26 additional parallel cultures kept in the dark but not shaken averaged 1710 worms. The character of the curves reflects these averages in the distribution of yield of the 3 groups of cultures.

<sup>4</sup> E. E. McCoy of the State of New Jersey Dept. of Agriculture has since informed me that summer temperatures of this range have been found deleterious for the nematode under field conditions.

This representative series indicated that unshaken cultures in the dark yielded about half, and cultures shaken in the light about three-fourths of the yield from cultures shaken in the dark.

The possibility that the advantages of the dark might be due to RLE being itself light-sensitive did not seem borne out in preliminary trials.

A glass tube of hog RLE exposed in the window to daylight for 4 days in Dec. 1950, was compared to a regular refrigerated sample of the same RLE in standard tests with veal infusion broth of pH 6.4 inoculated with 22 larvae and cultured in the dark-room shaker for 3 weeks. Two cultures with light-exposed RLE supplement had 19 and 30 hundred worms, average  $\times 111$  fold yield; two cultures with refrigerated RLE supplement had 26 and 35 hundred worms, average  $\times 139$  fold yield.

While this result is not considered definitive as regards the effect of light on RLE, it gave a working answer for conditions under which experiments at New York have been conducted. The light exposure tested was greatly in excess of what the RLE tubes receive in the laboratory transfer room, in which the window shade is always drawn, and artificial light used.

### III. MATERIALS AND METHODS

1. *Axenic cultures in fluid media.* Conditions that have come to be standard for routine testing (Stoll, 1951) include the use of test tubes  $22 \times 180$  mm. containing 10 ml. of medium (9 ml. of either veal or beef heart infusion broth supplemented with 1 ml. RLE, and 25 mg. dextrose), inoculated with (statistically) 25 infective 3rd stage larvae in 0.5 ml. water. The tubes are kept upright in a shaking machine in a dark room with temperature at about  $22^{\circ}$  C. Their initial 10.5 ml. volume ( $3\frac{1}{2}$  cm. depth) is reduced to 9 ml. by evaporation by the time they are examined for yield after 3 weeks incubation.

Sterility tests in infusion broth are made routinely on all components, including the worms, when cultures are initiated, and of individual cultures when they are examined, although at the latter time few contaminations are revealed that are not already visible.

2. *Broth.* Veal infusion broth as made at the Princeton laboratory of the Rockefeller Institute (Price, 1947) contained 1% peptone (Fairchild) and 0.5% NaCl. Beef heart infusion broth at the New York laboratory is made in substantially the same way, with Pfanstiehl peptone. Both show a nitrogen content of about 300 mg./%.

For *N. glaseri* cultures the stock broth is adjusted to appropriate acidity with normal HCl and re-sterilized in 200–300 ml. amounts in Erlenmeyer flasks by autoclaving at 15 lbs. for 15 min.

3. *RLE (Raw liver extract).* Liver, as from rabbit or hog, is secured promptly after killing the animal by exsanguination and chilled. Refrigeration for several days renders processing easier. If frozen, the tissue is thawed in the refrigerator overnight before use. Extracting and sterilizing by Seitz filtration are completed within an hour, if possible, and the temperature of the mixture preferably kept below  $21^{\circ}$  C. during the following steps:

A. One hundred grams liver is triturated in 400 ml. distilled water in a Waring blender for 5 minutes.

B. The brei with a resulting pH<sup>5</sup> of 5.9–6.4 is adjusted to pH 4.1–4.2 using normal HCl (9–12 ml. required).

C. Celite #503 is added at 10 g./% of the net volume and the mixture centrifuged at 2500 G<sup>6</sup> for 12 minutes. Centrifugation may be preceded by suction filtration through coarse paper in a Buchner funnel, and a lower gravitational field used. The precipitate, made up mostly of a firm plug, is discarded after pouring or pipetting off the clear supernate, which now has a pH of 4.3–4.5.

D. Sterilization of the supernate is effected with a chilled Seitz filter, using an S1 (or S3; Republic) sheet. In lots of 50–90 ml., filtration may require as little as 4–9 minutes at about 20 lbs. air pressure. Final pH is characteristically 4.4–4.6, but after refrigeration tends to shift toward pH 5.0.

<sup>5</sup> Using Beckman glass electrode pH meter.

<sup>6</sup> Produced at 4000 rpm in a centrifuge constructed by J. Blum of the R.I.M.R. It is a motor-drive, self-balancing, angle type (inclination of tubes  $30^{\circ}$  from vertical). Lusteriod tubes of 50 ml. are used. The gravitational force is calculated for the bottom of the tubes. A conventional type International centrifuge has also been employed but produces more heating at lower rpm and G.

Seitzed RLE represents 20 g./wt.% extract of original liver tissue, is at first serum-like in appearance, and contains about 300 mg./% N. In from one to several days at 2 to 4°C. refrigeration (more rapidly if warmed) a dark, fragile precipitate appears. In an undisturbed tube in the cold this is in the form of a cylindrical core. The resulting supernate is clear, usually light green, and represents  $\frac{1}{2}$  or more of the total volume. Before use, precipitate, if present, is resuspended.

Most of the activity of the extract accompanies the precipitate (see IV, 3), the amount of which depends in part on the promptness with which processing is completed. Thus, especially with a large batch, delay in Seitz filtration (as sometimes over the noon hour) may cause clogging of pads and eventual appearance of considerably less precipitate in the sterile filtrate later. Marked rise in temperature of the brei through incautious extension of the time in Waring blender or centrifuge results in similar loss (see IV, 4).

4. *Inoculation of cultures.* Axenic 3rd-stage larvae from a single storage lot are transferred to pointed and calibrated 15 ml. centrifuge tubes. Their number is adjusted per tube so that 0.5 ml. from the suspension, measured against the calibration of the tube, and withdrawn by Pasteur

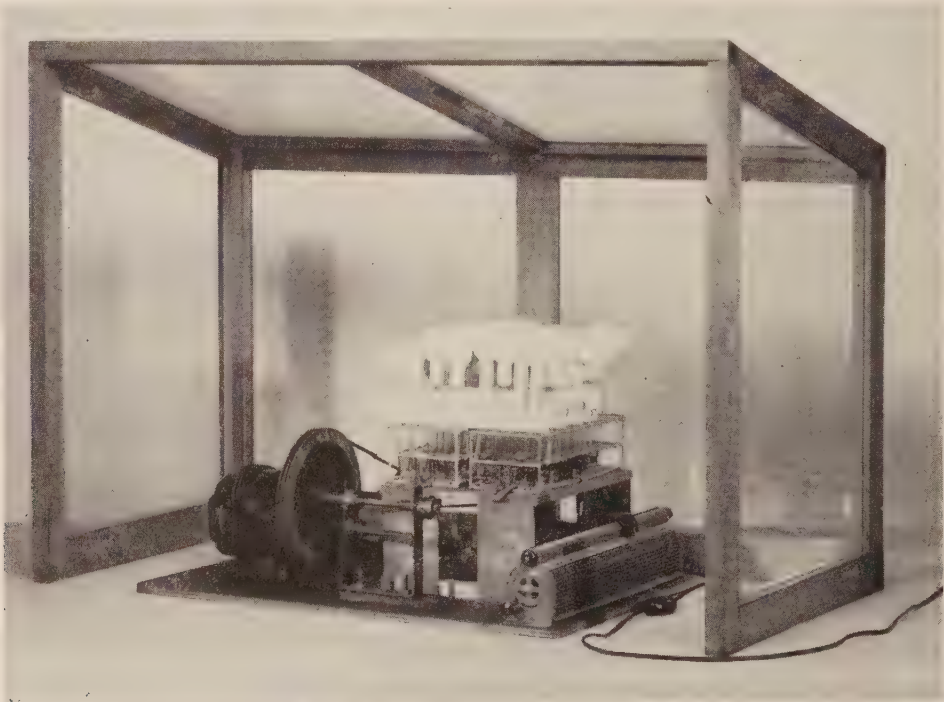


FIG. 5. Shaker used for *N. glaseri* cultures in fluid media. Capacity 80 tubes, traverse 10 mm. Photograph by J. A. Carlile.

pipette, contains the desired number of 25 active larvae. Tests show that the coefficient of variation on a series of withdrawals is about 30%. Due to the worms being poor swimmers they quickly sink, and great care is needed to keep them in homogeneous suspension. This renders the use of bacteriological transfer pipettes for sampling unsatisfactory.

A portion of the water in the tube containing the larvae is replaced by a sterile dextrose solution so that eventual transfer of 0.5 ml. of fluid with 25 larvae also contains 25 mg. dextrose.

5. *Shaking machine.* As noted (II, 6) yields have been larger when utilizing a shaking machine to agitate the cultures in the dark. The shaker most used has a traverse of 23 mm., is powered by a 1/16 h.p. motor, and holds racks with a capacity of 200 tubes. A smaller shaker with 10 mm. traverse and 80-tube capacity is illustrated in Fig. 5. Speeds of about 100 back-and-forth excursions per minute produce favorable results, and have been standard. Such speeds do not break the surface film in the culture nor do they agitate the media sufficiently to cloud the clear broth with the sediment from the RLE. Shaking at a rate of more than 300 times per minute has permitted development and multiplication although with the appearance of an appreciable number of teratological specimens.

The shaker, shielded by a hood (Fig. 5), is kept in a dark room that is temperature-con-

trolled. The nearly daily thermograph record since Sept. 1951, has shown a range of 21.1–23.3° C., with a mean of 22.5° C.

6. *Examination of cultures.* Some estimate can be made of whether conditions have been favorable in culture by examining them with the unaided eye or the low power binocular microscope. More reliable comparisons are made by determining the worm population of each culture. To do this a fair sample of about one ml. of culture is transferred with sterile precautions to a pointed, calibrated 15 ml. centrifuge tube with the aid of a Pasteur pipette. If the count is moderate, 0.5 ml. of an evened suspension of the particular volume in the centrifuge tube is withdrawn to a slide, and the pipette then rinsed in a tube of broth for a sterility test on the culture. If the count is high, a sub-dilution tube is made from the contents of the first tube (as by raising 0.5 ml. of the first to 5 ml.), from which in turn 0.5 ml. is taken for counting.

At 1 week, culture volumes approximate 10 ml., so that a 0.5 ml. fair sample contains 1/20th of the worms. At 3 weeks when the counts are higher and a 5 ml. sub-dilution tube is made, the slide count represents 1/10th of, say, 1/18th of the culture. If the culture has already been counted previously, as at one week by withdrawal of 1 ml. of medium, the number of worms in the aliquot of medium previously withdrawn is added to the 3-week count to represent the total yield of the culture. Duplicate counts are usually made.

Residual amounts of medium transferred from the culture and unused for counting are not returned. They are convenient for pH determination.

#### IV. RESULTS

1. *Culture yields in broth with and without RLE.* Table 1 summarizes an early experiment with veal infusion containing 10% hog RLE supplement, in contrast with unsupplemented broth.

TABLE 1.—*Yields of N. glaseri from individual 10 ml.-cultures in veal infusion broth without and with hog RLE. Third-stage larvae averaging 25 per tube inoculated 29 Nov. 1939, after harvesting from stock cultures on 7 Oct*

Day of experiment	Worm counts of individual cultures	
	Broth without RLE	9 ml. broth with 1 ml. RLE
4 <sup>a</sup>	14, 16 No Young	30, 35, 41, 51,
5	32, 38 No Young	16 (no young), 53, 252, 312
6	25, 31 No Young	167, 453, 583, 703
7	52, 68 <sup>b</sup>	154, 1120, 1300, 1800 <sup>b</sup>
21	112, 121, 167, 176 <sup>c</sup>	1830, 1880, 2300 <sup>c</sup>
Average fold yield @ 21 days	× 6	× 80

<sup>a</sup> 17 tubes examined on days 1–3 showed development proceeding to the adult stage but no young. They averaged 25.2 worms each. The 7 tubes labelled "no young" in the table average 24.6 worms each and illustrate variability in numbers of larvae inoculated.

<sup>b</sup> Two cultures with broth only had 23 and 32 adults, average 27; 4 cultures with RLE had 27, 42, 72 and 72 adults, average 53 per culture.

<sup>c</sup> Four cultures with broth only had 45, 64, 48 and 77 adults, average 58 per culture; 3 cultures with RLE had 714, 1015, and 1104 adults, average 944 per culture.

Individual tubes were chosen at random from the experiment on the given days, and discarded after examination. Young appeared characteristically on the 4th day in media with RLE, but not until 3 days later in broth alone. Veal infusion without supplement rarely produces more than 200 worms, few progeny becoming adult and reproductive.

As table footnotes indicate, there was a bare doubling of the number of adults between 1 and 3 weeks in unsupplemented broth. In broth with RLE the average count of adult worms at 1 week was already double the number of inoculated larvae, and this reached ×38 fold the inoculum in the average adult count at 3 weeks. This progressive increase in number of adults (56% of which were females in this experiment) indicated the worm populations were not due to the presence of giant forms, as occasionally found by McCoy, Girth and Glaser (1938) in infected beetle grubs.

Net yield here at 3 weeks averaged ×6 fold in broth, whereas tubes with RLE supplement reached ×80 fold through appearance of second, and perhaps third generation forms. In this experiment culture media were at pH 6.9–7.0.

Beef heart infusion broth, alone and with RLE, produces results comparable to the veal infusion broth above, and has been used interchangeably with it. Both have N content of about 300 mg./%. Early trials with Bacto tryptose phosphate broth supplemented with 6.7 g./% Bacto yeast extract (N content about 350 mg./%), and with a beef heart-hog stomach digest broth made at the Princeton laboratory (N content about 630 mg./%) gave good yields with 10 per cent RLE supplement under the conditions of experimentation then prevailing.

Bacto nutrient broth (N content 140 mg./%), which contains beef extract and peptone, has been unsatisfactory. Development has not been observed in it when unsupplemented, and only rarely has 10% RLE supplement with it produced even the initial development observed with unsupplemented veal or beef heart infusion broth. This failure of a broth made from an extract of beef muscle has a parallel in the failure of Glaser, McCoy and Girth (1942) to obtain development of either *N. glaseri* or *N. chresima* in an autoclaved, semi-solid gel medium made with beef muscle.

Failure of Bacto nutrient broth to permit development of 3rd stage larvae has interest also, as evidence that the capacity of *N. glaseri* larvae from stock kidney cultures to develop and multiply in appropriate fluid cultures, is not due to substances carried with them from the kidney cultures.

2. *Ratio of RLE supplement to broth.* Increasing amounts of RLE used with infusion broth show increasing yields in cultures examined after 3 weeks incubation.

In a typical experiment (Table 2) appreciable activity was demonstrated with as little as 0.05 ml. RLE (representing extractives from 10 mg. original liver), in 10 ml. medium. Smaller amounts of 0.01 and 0.02 ml. (*i.e.*, representing 2 and 4 mg. original liver) scarcely increased the average yield but did foster an improved condition of the worms, which were darker and more active than in unsupplemented beef heart infusion broth. Greater multiplication of the nematodes accompanied larger amounts of RLE, the usual 1:9 ratio with broth giving  $\times 127$  fold yield. The ratio of increase of the latter to 0.1 ml. RLE with 9.9 ml. broth is 3.9. In another experiment, where absolute yields were higher, this ratio was 3.3.

TABLE 2.—Yields of *N. glaseri* at 3 weeks from cultures, of beef heart infusion broth supplemented with rabbit RLE in graded amounts, at pH 6.1–6.2. Inoculation with 26 infective larvae per tube

Media (ml.)	Individual culture yields (hundreds)	Average yield	Fold yield
10 broth only	0.2, 1.6, 1.7	120	$\times 5$
9.99 broth, 0.01 RLE	0.3, 1.3, 4.1	190	$\times 7$
9.98 " , 0.02 "	0.7, 1.6, 2.9, 3.7	220	$\times 8$
9.95 " , 0.05 "	4, 4, 7, 11, 13	780	$\times 30$
9.9 " , 0.1 "	5, 6, 8, 15	850	$\times 33$
9.5 " , 0.5 "	18, 24, 30, 46	2950	$\times 113$
9 " , 1 "	13, 30, 40, 49	3300	$\times 127$
8 " , 2 "	28, 31, 40, 42, 48	3780	$\times 145$
10 RLE <sup>a</sup> only	32, 41, 41, 71	4630	$\times 178$

<sup>a</sup> Rabbit RLE, pH 4.6, titrated under sterile conditions with N/1 NaOH to pH 6.2 (except the 4th tube to pH 6.6), and 0.5 per cent NaCl added. Despite the high yield, the "RLE only" tubes showed one striking deficiency as compared to all the others, in that only 36 per cent of the worms were alive at 3 weeks; in the 30 other tubes, 80 to 92 (average 85.5) per cent were alive.

This experiment also illustrates that RLE, contrary to early experience, can be used as a medium without broth (although extracts made similarly from kidney have not been successful). Here after titration to pH 6.2 (and 6.6) and addition of 0.5% NaCl (with 25 mg. dextrose per tube, as routinely added with inoculum), RLE gave good results. The condition of the worms, however, was not so good at 3 weeks, only 36 per cent being alive in contrast to 80 to 92 per cent in the broth tubes supplemented with RLE. A further contrast in this experiment was that no exsheathing had occurred at 3 weeks in tubes with 10%, 20%, and 100% RLE, but at  $\frac{1}{2}$ %, 1%, and 5% RLE supplement about two-thirds of the dauer larvae were casting their sheaths.

3. *Activity of RLE precipitate.* After sterilization by Seitz filtration, RLE has the appearance of a slightly opalescent serum. On storage in the refrigerator, a

loose, clot-like precipitate appears, leaving a clear green supernate which retains 90% of the N content of the original RLE.

In Table 3 the results are shown of contrasting such a precipitate and its supernate, in relation to the original rabbit RLE (after 5 weeks refrigerator storage) as supplements with beef heart infusion broth, and in relation to broth unsupplemented. The supernate was re-Seitzed to free it of all flecks of precipitate. For test the precipitate itself was resuspended in a minimum of supernate, representing removal of 3/4 of the supernate volume present in the original RLE. After 3 weeks incubation the fold yield of supernate as supplement to broth was  $\times 46$ , and of precipitate  $\times 172$ , or nearly quadruple the activity. Original RLE as supplement yielded  $\times 151$  fold, broth alone  $\times 5$ . Use of supernate seemed to increase the number of abnormal and "warty" young worms, not infrequently observed at 3 weeks in unsupplemented broth and other deficient media.

TABLE 3.—*Contrast in activity for N. glaseri multiplication between the precipitate that appears after storage of RLE, and its supernate. Yields at 3 weeks are shown from tubes of 10 ml. beef heart infusion broth alone and 9 ml. broth supplemented with 1 ml. rabbit RLE, its precipitate, or its supernate (re-Seitzed to remove traces of precipitate). Inoculation with 25 infective larvae per tube*

Media	Individual culture yields (hundreds)	Average yield	Fold yield
10 ml. broth (pH 6.1)	0.4, 0.9, 1.4, 2.3	120	$\times 5$
9 ml. broth, with 1 ml. RLE supernate (pH 5.1) <sup>a</sup>	2, 13, 15, 16	1150	$\times 46$
9 ml. broth, with 1 ml. RLE precipitate (pH 4.7) <sup>b</sup>	38, 42, 42, 50	4300	$\times 172$
9 ml. broth, with 1 ml. original RLE (pH 4.6) <sup>b</sup>	32, 36, 37, 46	3770	$\times 151$

<sup>a</sup> Resulting pH 6.1.

<sup>b</sup> Resulting pH 6.0.

In a test, not detailed, with another rabbit RLE, a precipitate as supplement gave  $\times 225$  fold yield, or more than 10 times its supernate similarly tested which gave  $\times 20$  fold yield. In this instance there was no unsupplemented broth control, but broth, pH 6.5, with original RLE, pH 4.9, of 3 weeks refrigerator storage yielded  $\times 167$  fold.

4. *Heat lability of RLE.* Many early failures to secure a raw liver extract that was an effective supplement with infusion broth for the culture of *N. glaseri*, as well as variability in the effectiveness of successive extracts, were later found associated with rise of temperature in processing RLE. The following experiments illustrate the heat lability of the supplement, in relation to the results before Seitzing, and afterward.

A. *Heating before Seitz filtration.* When making hog RLE on 11 May 1951, the brei came from the Waring blender at pH 6.0 and a temperature of 15° C. An aliquot of this was processed in the usual way, and after Seitz filtration had a pH of 4.4.

The balance at pH 6.0 was divided into two portions; half of it was acidified to pH 4.1; each of these portions was in turn subdivided into three equal parts. One aliquot at pH 4.1 and one at pH 6.0 were incubated at 37° C. for 5 hours; similar paired portions were tubed and heated for 4 hours in a water bath at 56° C.; and the remaining thirds were autoclaved at 15 lbs. pressure for 15 minutes. These 6 heated portions were then separately filtered through paper to remove the coagulum. The three pH 6.0 lots were acidified to pH 4.2, and all six Seitz-sterilized. The resultant extracts were pale green, the paling accentuated somewhat in ratio to the higher heat exposures, and pH of 4.6–5.1. They developed no precipitates later.

On 16 May the six heated portions and the regular RLE were put into test with veal infusion broth in the usual ratio of 9 ml. broth and 1 ml. supplement at pH 6.4. When examined after 3 weeks incubation in the shaker, five cultures with regular RLE had yields of 11, 16, 16, 27, and 30 hundred worms, average 2000. Of 24 cultures supplemented with the aliquots heated before Seitz filtration (whether before or after taken to pH 4.1–4.2), 21 were failures (*i.e.*, less than 100 worms per tube), and 3 had yields of 160, 230 and 310 worms respectively—a result not different than with infusion broth unsupplemented.

This is the usual result in heating before filtration for shorter periods as well,

loss of activity accompanying the removal of the coagulum. In one instance, however, heating at 37° C. for 4 hours before filtration failed to remove all the activity (although 2 hours at 56° C. did), eight cultures on standard test showing an average of 750 worms.

B. *Heating after Seitz filtration.* When making hog RLE on 21 June 1951, the brei which came from the Waring blender at 18° C. was processed in the usual way, Seitz filtered, and showed a pH of 4.4.

Five samples chosen at random of the sterile RLE were handled as follows: one was refrigerated as usual (4° C.), one left on the laboratory table at 29° C. (19 hours), one incubated at 37° C. (19 hours), one placed in the water bath at 56° C. (19 hours), and one autoclaved at 15 lbs. for 15 minutes. They were then used as supplements in a standard test experiment with veal infusion broth of pH 6.5. When examined after 3 weeks incubation in the shaker, five tubes with the usual refrigerated RLE yielded 13, 16, 20, 20, and 21 hundred worms, average 1800; five with RLE at room temperature 11, 13, 14 and 16 hundred worms, average 1350, with one failure; five with RLE incubated at 37° C. yielded 3 and 6 hundred worms, with three failures; five with RLE heated at 56° C. yielded 4, 6, 6, and 7 hundred worms, with one failure; five with autoclaved RLE yielded five failures; and five control tubes with veal infusion broth yielded five failures.

The balance remaining of these RLE samples were retested a month later: 3 tubes with continuously refrigerated RLE averaged 2000 worms, 3 tubes with RLE held continuously at room temperature (range 27.8–32.8° C.; 82–91° F. thermograph) averaged 600 worms, and 4 tubes with RLE held continuously at 37° C. averaged 670 worms.

A result with rabbit RLE 5, of pH 4.7, was confirmatory of the loss of activity after long exposure to room temperature. It was tested under standard conditions 22 May 1951, with one sample that had had refrigerator storage at 2–5° C. for 6½ months, and a second sample that had been on the laboratory table top at room temperature (average 26.7° C., thermograph 76–83° F.) for 5½ months. Yield at 3 weeks of tubes supplemented with refrigerated RLE was 34, 36, 40, 43, and 59 hundred worms, average 4240, contrasted with room temperature RLE supplement tubes giving 9, 11, 13, 18, and 21 hundred worms, average 1440.

On the other hand, short exposures at 37° C. of Seitz-sterilized RLE have not always shown loss of activity. On 25 Sept. 1951, a sample of hog RLE 20, of pH 4.7, held at 37° C. for 5 days after Seitzing, was compared with a regular refrigerated sample as a supplement under standard conditions. Yield at 3 weeks of tubes with refrigerated RLE was 20, 25, 25, 27, and 31 hundred worms, average 2560, as compared to tubes with RLE at 37° C. for 5 days with 16, 20, 29, and 35 hundred worms, average 2500.

5. *Extracts from other sources.* Besides RLE prepared from hog and rabbit livers those from freshly killed sheep and ducks have shown as supplements average activities of about  $\times 100$  fold yield of worms in standard culture tests. Unsatisfactory has been RLE from commercial frozen chicken livers, with less than half this much activity.

The latter range of activity was obtained also with an aqueous extract of horse liver powder Viobin (labelled "desiccated and defatted at 37° C."), using 37 g. powder as the equivalent of 100 g. fresh liver. Other liver powders (Viobin 40°, Viobin 75°, Difco Bacto, N.B.C. Liver Fraction L containing the alcohol-soluble fraction of liver, and N.B.C. Fraction 2 containing the alcohol-insoluble fraction of liver) have not produced useful extracts. In most cases tests of their activity showed no improvement over infusion broth alone.

Extracts prepared similarly from fresh kidneys have not exhibited more than a fraction of the activity of RLE, although processing for the extract is easier. From calf, hog, sheep and rabbit, freshly prepared raw kidney extracts used as supplements with infusion broth in standard experiments gave averages of less than  $\times 50$  fold yield of worms. These extracts showed little or no precipitate after storage, and had an N content of 120–160 mg./%.

Extract of pH 4.6 prepared from 16-day rabbit embryos, including their amniotic fluid, has proved a good supplement. In this instance no precipitate occurred in the culture tubes, indicating the worms are not dependent on particulate food.

6. *pH range.* Early encouragement of development in liquid media had come from observing the production of a few adults in veal infusion broth of pH 7.2–7.4. It was then found that some development would occur over a wide pH range, from less than 5 to more than 9. Several tests designed to determine the optimum range turned out rather unsatisfactorily, but did indicate that RLE-broth media with a pH of 6.0–6.5 were more productive than when neutral or alkaline. This pH range,

secured by using infusion broth of pH 6.1–6.5 with RLE of pH 4.4–5.0 was accordingly used as standard.

Recent tests have indicated even better yields of worms occurring at a slightly lower pH, as shown in Fig. 6. In this experiment there was no multiplication at

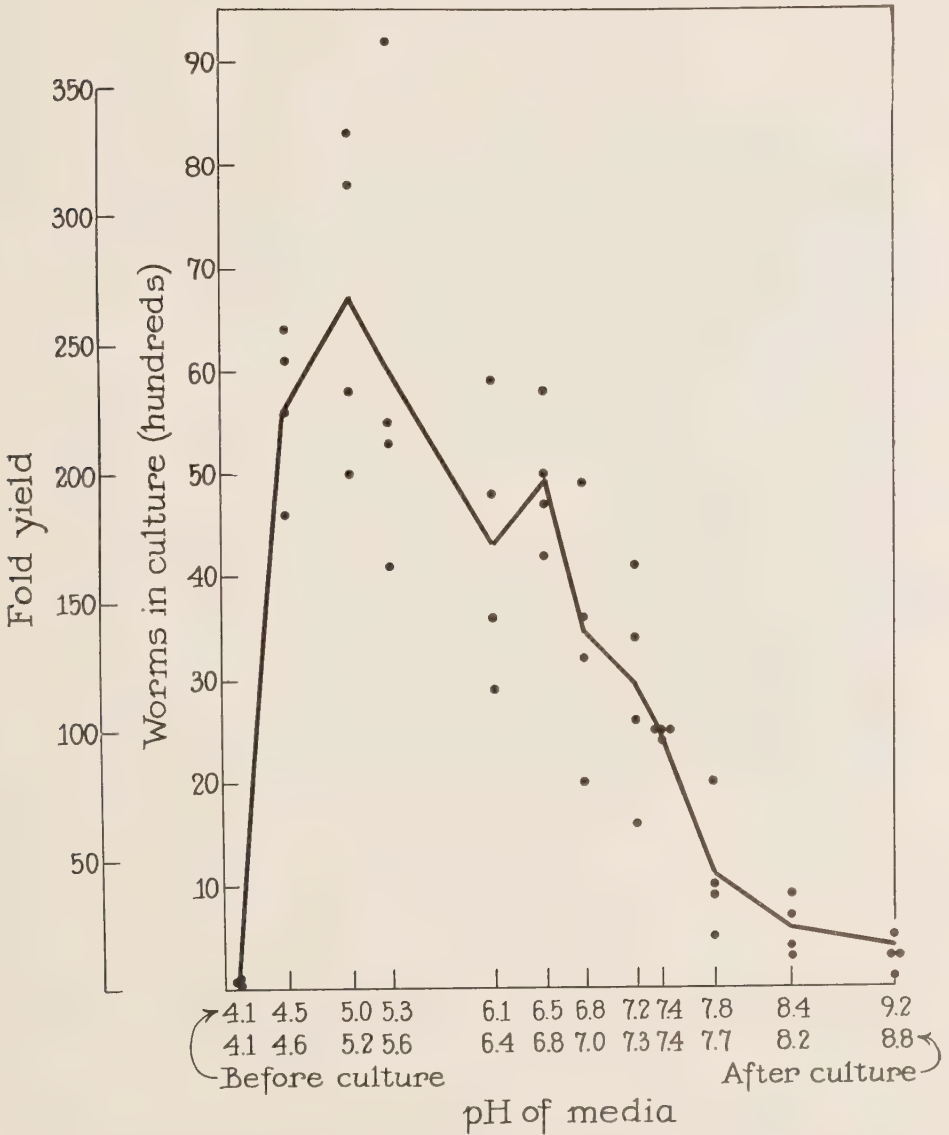


FIG. 6. Experiment to illustrate yields of *N. glaseri* in relation to pH. Cultures (9 ml. beef heart infusion broth of various pH values, supplemented with 1 ml. rabbit RLE, pH 5.0) inoculated with 25 infective larvae and shaken in the dark for 3 weeks.

pH 4.1, but at 4.5–5.3 averaged over  $\times 200$  fold yield. This was maintained at nearly  $\times 200$  at 6.1 and 6.5, but rapidly declined beyond 7.0. Fig. 7 illustrates worm stages in a culture begun at pH 6.1.

7. Culture "failures." No inconsiderable degree of reliability can now be an-

ticipated with the *N. glaseri* cultures. For a dioecious metazoan form this means not only growth and development, but multiplication—the physico-chemical opportunity for successful mating and reproduction.

In testing various factors in relation to goodness of culture conditions, and employing the relatively small inoculum of 25 infective larvae, occasional cultures have failed to produce good yields even when companion tubes with similar make-up and handling have done so. Based on an analysis of 1023 recent cultures, these “failures” turn out to be related to the over-all goodness of culture conditions. Thus when

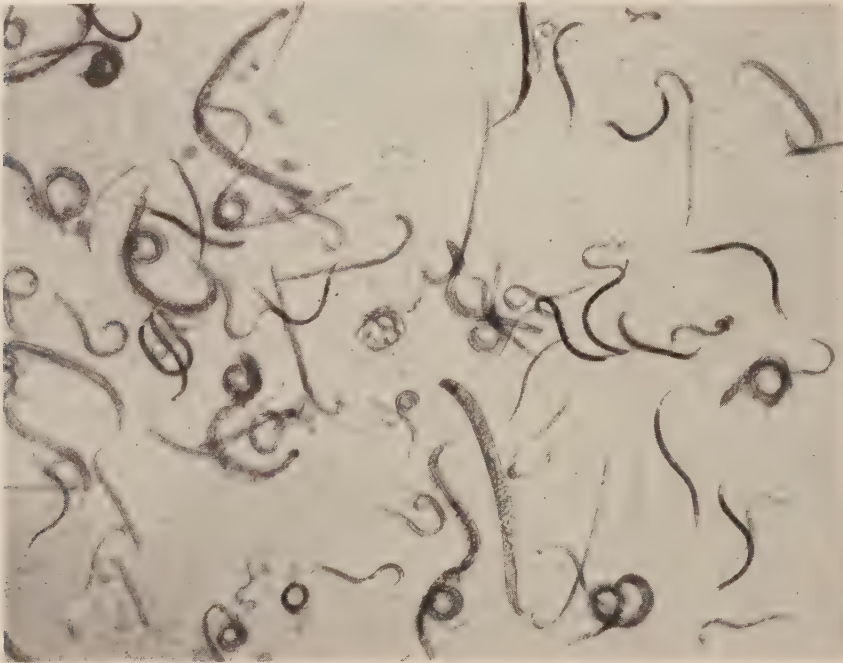


FIG. 7. *Neoapectana glaseri* after 3 weeks culture in axenic fluid medium, pH 6.1, of 9 ml. beef heart infusion broth supplemented with 1 ml. rabbit RLE. Among various stages visible—including adult males and females, alive and spent—are the re-appearing, black, “dauer,” 3rd stage larvae. Mag.  $\times 20$ , in culture medium, no coverslip. Photograph with Kodatron speedlight (1/10,000 sec.) by R. F. Carter.

companion cultures in a clutch of tubes supplemented with the same RLE yielded more than 1250 worms in 3 weeks, the failures (*i.e.* tubes yielding less than 100 worms) were 27% ; when parallel yields were between 1300 and 3750, failures were reduced to 6% ; when yields exceeded 3750, failures were less than 4%.

From these results it is concluded that occasional inocula low in numbers of infective larvae in a dilution drop designed to contain 25 larvae, or with an initial lopsided sex ratio, fail to establish good populations under poor culture conditions, but are able to overcome an early statistical handicap if the sum of culture conditions is favorable.

The above refers to axenic cultures, the individual sterility of which has been tested. Omitted from consideration are the discards due to contaminations, about 1/3 of which are molds, frequently an individual colony from a single spore that escaped sterilization or aseptic procedures, and revealed itself the 2nd or 3rd week.

Mold growth is favored by the room temperature-3 week culture regimen, and would probably escape notice in shorter incubation periods at higher incubator temperatures. Contaminations from all causes in the last 2000 cultures studied at Princeton dropped from 17% in the first, to 7% in the second. After a thousand cultures at New York showed 10% contaminations, a second thousand showed reduction to  $4\frac{1}{2}\%$ .

#### V. COMPARISON OF *N. glaseri* ON KIDNEY TISSUE AND IN FLUID CULTURES

1. *Yields of worms.* Glaser, McCoy and Girth (1942) described an autoclaved medium that gave excellent growth with *N. chresima* "and may be used for *N. glaseri* as well." This was "a semi-solid gel at pH 7.0, in the proportion of 20 g. ground beef kidney or liver, 100 ml. water, 0.5 g. sodium chloride, and 0.5 g. agar." They reported yields on the 23rd day from 50 ml. of the medium in flasks inoculated "with approximately 10,000 infective" *N. glaseri* of 538,000 with beef liver base and 428,000 with beef kidney base, respectively  $\times 54$  and  $\times 43$  fold yield.

Tests of this in 1950, using ground hog-kidney base, did not give satisfactory results in large test tubes containing 15 ml. media (5 cm. deep) inoculated with 26 and 90 larvae of *N. glaseri*. Due to the gel they did not seem adaptable for dilution counting, but estimated yields did not exceed expectation with unsupplemented veal infusion broth.

However, tubes of this semi-solid gel with 1 ml. of rabbit RLE added gave distinctly increased worm populations.

This result was not dissimilar to a test made with autoclaved hog liver brei, pH 6.4, when used as a 10% supplement with veal infusion broth of pH 6.5 in a standard test experiment. There was an average of only 36 worms in four "failure" cultures, whereas the control employing regular RLE supplement made from portions of the same tissue gave  $\times 75$  fold yields.

It has also been found that autoclaved whole kidney tissue lost its characteristic capacity for growing *N. glaseri* on dextrose agar slants. This suggested the addition of RLE to pieces of autoclaved kidney as a substitute type of "stock" cultures. Results were excellent.

The following experiment was accordingly done.

The kidneys of a 3.2 kg. exsanguinated female rabbit were aseptically removed, decapsulated, and each cut into 8 pieces of approximately 1 g. size. Six of the one-gram portions were transferred to separate dextrose agar slants and the remaining 10 pieces autoclaved at 15 lbs. for 15 min. before transfer in turn to similar slants. To each of 5 of the latter was added initially 0.3 ml. rabbit RLE (more RLE being added at intervals throughout the experimental period), the remaining 5 left without RLE supplement. All the tubes were then inoculated with 100 infective larvae each. As comparisons, 20 tubes of 9 ml. veal infusion broth were supplemented with 1 ml. each of the same RLE, and half of these inoculated with 100 larvae, half with 25. The tissue tubes were placed in a tray in the dark room, and the fluid cultures in a shaking machine for the culture period. Tubes from each group were then chosen at random for parallel examination and worm count on various days of the experiments. Those examined at 3 weeks reflected comparative conditions of earlier examinations and were as follows:

A. Three dextrose agar tubes prepared with one-gram pieces of raw kidney tissue yielded respectively 48,600, 80,100, and 89,400, or an average of 72,700 worms. Of these, 38% were recovered from the walls of tubes, 54% from surface of kidney and slant, and 8% were in the agar.

B. Two dextrose agar tubes prepared with 1-gram pieces of autoclaved kidney, to which RLE had been added at intervals to a total of 1.4 ml. each (representing 280 mg. original liver) yielded respectively 16,200 and 47,300 or an average of 31,700 worms. Of these, 10% were recovered from the walls of the tubes, 72% from tissue and slant, and 18% were in the agar. The small numbers from glass suggested migration had not yet reached its peak.

C. Two dextrose agar tubes prepared with 1-gram pieces of autoclaved kidney unsupplemented yielded respectively 700 and 900 worms. These were all from surfaces of tissue and slant, none being observed on glass or in agar.

D. Three fluid cultures containing 1 ml. RLE (representing 200 mg. original liver) and inoculated with 100 larvae each (as were A, B, and C) had respectively 2600, 4100, and 5500, an average of 4070 worms.

E. Three fluid cultures inoculated with 25 larvae each, had respectively 1600, 2300, and 3200, an average of 2370 worms.

Insofar as this experiment is representative, fluid cultures do not yet permit development and multiplication comparable to that on raw kidney tissue. The A/D ratio is 18; the A/E ratio is 31. On the other hand, RLE substantially restores to autoclaved kidney, B (which unsupplemented, C, was a failure as a culture medium) its capacity to permit *N. glaseri* to thrive, the A/B ratio of total yield being slightly over 2.

The raw kidney tissue in A produced in 3 weeks at the rate of 13.8 mg. kidney per 1000 worms. The autoclaved kidney tissue in B supplemented by an amount of RLE equivalent to 280 mg. original liver, produced in 3 weeks at the rate of 8.8 mg. original liver per 1000 worms. These are not strikingly dissimilar values. However, in the fluid cultures, those inoculated with 100 larvae, D, produced at the rate of 49 mg. of original liver (in RLE added) per 1000 worms, and those inoculated with 25 worms, E, at the rate of 84 mg. original liver (in RLE added) per 1000 worms. (Standard cultures yielding 2500 worms, or  $\times 100$  fold the inoculum of 25 larvae, would have had available 80 mg. original liver in RLE added, per 1000 worms.)

From this experiment it may be concluded that RLE renders available to worm populations on autoclaved kidney on dextrose agar slants, about the same amount of necessary components as are made use of in raw kidney tissue in 3 weeks. The fluid cultures did not demonstrate as efficient use of the RLE. This may be due in part to the presence in autoclaved kidney of materials needed by *N. glaseri* and unavailable in the present infusion broth. It may be due in part to the physico-chemical differences in culture habitats at the moist interface of tissue as compared to the bottom of a 3.5 cm. column of fluid, although cultures of twice or thrice this depth show about the same yields.

Use has been made of autoclaved kidney plus RLE for serial stock cultures when fresh sterile kidney is temporarily unavailable. RLE has also been an efficient supplement for raw sterile kidney that has been stored too long to permit multiplication of *N. glaseri*.

2. *Serial cultures.* In one other way the fluid cultures to date have shown a difference in result from kidney cultures, the latter being readily employed as stock cultures for serial transfer, but not the former. A large percentage of worms surviving in fluid cultures at and after 3 weeks are 3rd stage dauer larvae, but up to now little success has been had in maintaining fluid cultures in series, culturing from one to another. Inasmuch as dauer larvae from fluid cultures transfer back to kidney and establish thriving cultures, the possibility presents itself that a technical procedure allowing them to migrate to a moist surface before transfer to another fluid medium may be necessary. Occasionally, in just initiated stock cultures in which the larvae are caught in a small volume of water at the base of the tube, migration to the surface of the kidney appears to await sufficient evaporation of the

water to permit them to move in a capillary film, as is familiar with hookworm larvae.

#### VI. A CYCLE OF FLUCTUATION OF YIELD IN STANDARD CULTURES

With the use of a standard axenic culture procedure, *i.e.* 9 ml. broth supplemented with 1 ml. RLE and 25 mg. dextrose at pH 6.0–6.5, inoculated with 25 infective larvae and shaken for 3 weeks in the dark at mean temperatures of 24.1° C. (Nov. 1950–Aug. 1951) to 22.5° C. (Sept. 1951–Oct. 1952), average yields of 2500 ( $\times 100$  fold) or better are expectation. Table 4 illustrates such a result in an Oct. 1952 experiment (presented in its entirety) when 7 rabbit RLE of 2½–6½ months storage were cross checked for activity. Here the mean for all tubes was 3710 worms, or  $\times 137$  fold yield, and only 2 of the 35 tubes showed less than  $\times 100$  fold.

TABLE 4.—*A cross-check of seven successive rabbit RLE preparations, pH 4.8–5.0, each tested by 5 tubes, using 1 ml. supplement with 9 ml. beef heart infusion broth, pH 6.1; resulting media pH 6.0. Yields of N. glaseri after 3 weeks incubation following inoculation with 27 infective larvae on 6 Oct. 1952*

RLE and age (months)	Individual culture yields (hundreds)	Average yield	Fold yield
A 6½	24, 36, 37, 49, 51	3940	$\times 146$
B 6	23, 36, 29, 32, 33	2860	$\times 106$
C 5½	32, 33, 34, 34, 40	3460	$\times 128$
D 5½	29, 30, 35, 35, 50	3580	$\times 133$
E 5	38, 39, 42, 50, 51	4400	$\times 163$
F 2½	32, 35, 36, 37, 40	3600	$\times 133$
G 2½	32, 34, 39, 48, 53	4120	$\times 153$

There was a period of several consecutive months when such good results were not obtained. In Table 5 are summarized the yields of the 680 control cultures made with hog and rabbit RLE and either veal or beef heart infusion broth, at pH 6.0–6.5, since Nov. 1950, when the temperature-controlled incubation room became available. Average yields as well as the percentage of cultures greater than  $\times 100$  fold are indicated. Cultures made with both hog and rabbit RLE had high average yields during the fall, winter and early spring of 1950–1951 (HA, RA, HB, RB in table). This was followed by a waning period from late spring of 1951 through summer, winter and early spring of 1952 (HC and RC), followed in turn by a new, current period of high yield (RD).

TABLE 5.—*Summary of 680 control cultures examined at 3 weeks incubation for the period Nov. 1950–Oct. 1952. From May 1951 to March 1952 occurred a period of unsatisfactory yield without known cause. Cultures before and after this time were higher in average yield, and in the percentage showing greater than  $\times 100$  fold multiplication*

Group*	RLE prepared	No. of cultures	Average yield	Per cent of cultures with greater than $\times 100$ fold yield	Cultures begun
HA	Oct. '49–May '50	30	4240	80	Jan. '51–May '51
RA	Dec. '49	15	4780	80	Jan. '51–May '51
HJB	July '50–Feb. '51	107	2970	51	Nov. '50–April '51
RB	Nov. '50–May '51	82	3750	76	Dec. '50–May '51
HC	May '51–Sept. '51	142	1630	17	May '51–Jan. '52
RC	Oct. '51–March '52	127	2130	30	Oct. '51–March '52
RD	April '52–Oct. '52	177	3370	82	April '52–Oct. '52

\* RLE source: H, hog; R, rabbit.

RLE tested up to 7 months after preparation, except HA at 7½–19 months and RA at 13–18 months. (RA re-tested in Oct. '51 at 23 months after preparation averaged 4430 on 4 cultures).

The cycle of depression in culture yields occurred despite maintenance and scrutiny of the standardized experimental procedures. All glassware (except the culture tubes) receives a distilled water rinse before final sterilization as routine procedure. Back-checking of possible changes in infusion broths, larval stocks, and other factors furnished no consistent explanation of this variation. There was, however, an association of the cycle with certain RLE preparation periods, despite no change in source of hog or rabbit livers.

Results of cultures made in May 1951 illustrate this. In that month two newly prepared hog RLE (HC group) gave cultures poor in yield, although other RLE were producing satisfactory yields. Among the latter were one hog RLE (HA group) and one rabbit RLE (RA group) prepared in Princeton in Dec. 1949 and now 18 months old, and one rabbit RLE (RB group) prepared in May 1951 and tested the same month. Six additional hog RLE (HC group) prepared through Sept. 1951, and six rabbit RLE (RC group) prepared from Oct. 1951 to March 1952 continued, with minor variations, to produce cultures showing this low yield phase.

No new hog RLE has been prepared since Sept. 1951, but cultures made with rabbit RLE preparations since April 1952 have again shown satisfactory yields.

#### VII. INFECTIVITY OF CULTURE STRAIN OF *N. glaseri* FOR JAPANESE BEETLE GRUBS

In view of the long period the culture strain had passed axenically *in vitro*, encompassing an estimated 201 generations, the question arises as to whether it has lost its infectivity for its insect host. Tests in Oct. 1951 of 3rd stage larvae harvested in March 1950 and July 1951 (respectively from cultures of the 16th and 21st transfers since February 1948) showed the nematodes to have retained ability to parasitize Japanese beetle grubs (Stoll, 1953). Deaths of some grubs were associated with heavy infections of the nematode.

#### VIII. DISCUSSION

1. The idea that a heat-labile extract prepared from fresh liver would be an effective supplement for *in vitro* cultivation of a parasitic nematode such as *N. glaseri* grew out of an earlier problem. Glaser and Stoll (1938a) had shown that free-living stages of *Haemonchus contortus* could be sterilely grown from egg to infective stage in a medium of dilute agar, commercial liver extract (non-protein, heat-stable), fresh sterile rabbit kidney and heat-killed ground yeast; also (Glaser and Stoll, 1938b; 1938c) that 3rd stage larvae of *H. contortus* grown as above, or alternatively grown in fecal cultures and then exsheathed and sterilized (Glaser and Stoll, 1940), would develop to the last part of the 4th larval (2nd parasitic) stage *in vitro* in a medium similar to the above, with the addition of sheep blood, and substitution of commercial extract by one made from fresh sheep liver (Glaser and Coria, 1933).

Having secured this much result, which in a few instances yielded even young *Haemonchus* adults, when occasion came to repeat and extend the work in another laboratory year, not even a duplication of the earlier degree of development could be procured. The reason for this failure defied determination and was suspected of being in the liver extract component. It had been our experience that extracts from fresh sheep livers were substances troublesome to process and sterilize by

filtration (as by Berkefeld N candles then being employed) unless previously heated, at least cautiously, on a water bath to separate the coagulum.

As a consequence, extra effort was expended in the present work to secure an extract from liver that could be sterilized by filtration (Seitz) without previous heating.

This effort to find in RLE an effective heat-labile supplement proved a useful one. Thriving fluid cultures of *N. glaseri* producing successive generations and  $\times 100$  to  $\times 300$  fold yield from an original inoculum of 25 3rd stage larvae can be secured. Its success served to emphasize two aspects of the problem with *N. glaseri*. One involves growth and development of larvae to the adult stage, and some minor production of young. The other involves replication in turn of the reproductive cycle by the young forms. For *N. glaseri*, heat-stable veal or beef heart infusion broth is capable of allowing development and production of a few young, a portion of which in turn may even struggle through a non-normal adulthood. Heat-labile component(s) in RLE, supplementing the heat-stable broth, provide one type of favorable environment in which multiplication of the organism takes place freely.

The logical possibility needs consideration that all necessary substances for multiplication may be present in heat-stable infusions, but at less than a threshold concentration, as compared to RLE. On the other hand, the fact that RLE used alone (Table 2) allowed satisfactory multiplication of *N. glaseri* indicates that its method of preparation retains a moiety of necessary heat-stable substances as well. Dougherty and Calhoun (1948) reported some multiplication of the free-living nematode, *Rhabditis pellio*, in liver extract alone.

The experiment given in Table 1 illustrates the difference in value of heat-stable and heat-labile components. Veal infusion broth without RLE, inoculated with 25 infective larvae, averaged at 7 days 60 worms per culture, of which 27 were adults; and at 21 days had the small average yield of 144 worms with 58 adults per culture. The latter population can be accounted for by an average yield per female (of worms developing from the inoculated larvae) of about 9 or 10 offspring, a few of which were able to develop as far as adults in turn, but not to reproduce. When, however, heat-labile RLE was added to the heat-stable infusion broth and tubes were similarly inoculated from the same pool of larvae, worm populations at 1 week averaged 1090 worms per culture, of which 53 were adults. It is known that 7-day-old cultures with RLE supplement may include offspring from worms born and developed to sexual maturity in the culture tube, *i.e.*, second generation. By 21 days the 3 tubes in this experiment with RLE supplement averaged 2000 per culture, of which 944 were adults. It is evident that multiplication here was made possible with heat-labile component(s) from RLE.

Lawrence (1948) in Sydney, Australia, has recently cultivated aseptically the free-living stages of *Ancylostoma braziliense*, from eggs sterilized in 10% formalin in 10% "Miltions" solution. He reared the larvae to infective stage with the dilute agar—commercial liver extract—fresh sterile rabbit kidney—ground yeast medium used by Glaser and Stoll (1938a) in their earlier study with *Haemonchus contortus*. In an extension of the work Lawrence found that ground yeast and commercial liver extract "could be omitted without stopping the larvae from reaching the 3rd stage," but that the "essential ingredient in Glaser and Stoll's medium is the fresh sterile rabbit kidney;" also, and oppositely, that "just as good results were usually obtained with autoclaved kidney media as with those containing the fresh tissue."

Stoll (1940) tested specifically for those *in vitro* conditions favoring ecdysis at the end of the 3rd larval (1st parasitic) stage of *H. contortus* and production of 4th stage larvae. He

found commercial liver extract (heat-stable) added to diluted Ringer or Tyrode's solution at lowered oxygen tensions and at sheep temperatures, furnished the necessary stimuli. There was no further growth of the numerous 4th stage larvae that reached the latter transition, even when they were added to nutrient-rich media.

Von Brand and Simpson (1944) had one specimen of a larval stage (3rd?) of the nematode *Eustrongylides ignotus*, removed with sterile precautions from its cyst in *Fundulus heteroclitus*, molt into a young (4th stage?) male, after being incubated in 1.6% Bacto-broth (heat-stable) containing 0.85% NaCl, and 0.5% glucose.

Weinstein (1949) cultivated free-living stages of *Ancylostoma caninum*, *A. duodenale*, and *Nippostrongylus muris* "in the absence of living bacteria . . . using either fresh chick embryo or rat liver extracts containing penicillin and streptomycin." Heating rat liver extract inactivated it. His detailed methods are not given, but "extracts passed through a Seitz filter completely lost their ability to stimulate growth." Glaser and Stoll (1938a) and Weinstein (1949) demonstrated that 3rd stage larvae thus grown bacteria-free from eggs would infect their appropriate sheep and dog hosts normally.

Weller (1943) obtained development of *Trichinella spiralis* in roller tube cultures. He transferred 3rd stage larvae isolated bacteriologically sterile from infected muscle, to cultures prepared by planting fragments of minced 8 to 10-day-old chick embryo tissue in a chicken plasma clot, overlaid with 1.6 ml. nutrient fluid composed of 7 parts of Simms' solution, 2 parts chicken embryonic extract and one part chicken serum. Some larvae "developed to the stage of sexual differentiation," but whether they went beyond the 4th stage is not clear.

With the free-living *Rhabditis pellio* and *R. elegans* (= *R. briggsae*) rendered axenic with antibiotics, Dougherty and Calhoun (1948) obtained growth and reproduction for several generations when Seitz-sterilized liver extract was added to a complex medium. Dougherty (1950) later termed the heat-labile substance (or substances) required by *R. briggsae* "factor Rb" and has found the "factor" also in chick embryo juice (both fresh and lyophilized commercial sources), and in human whole blood and plasma.

With the nematodes, therefore, heat-stable factors have been found utilizable for sterile development of *Ancylostoma braziliense* from egg to 3rd larval stage, of *Haemonchus* from 3rd to 4th larval stage, of *Eustrongylides* from 3rd? to 4th? stage, and for *N. glaseri* from 3rd to adult stage with production of some young. On the other hand, heat-labile supplements, variously present in kidney, liver, testis, chick and rabbit embryo, and human blood appear to be requirements for development of free-living larval stages of such nematode parasites of mammalian hosts of *Haemonchus*, *Ancylostoma*, and *Nippostrongylus*, and for parasitic stages of *Haemonchus* and *Trichinella*; for multiplication through successive generations of *N. glaseri* of insect grub hosts; and for multiplication of the free-living soil nematode *Rhabditis*. Whether these supplements are identical for the different genera and species awaits further investigation.

2. The demonstrated ability of *N. glaseri* to multiply axenically both on kidney tissue, as devised by Glaser (1940b), and in fluid media, makes consideration of its parasitic status of interest.

The culture strain has clearly retained its capacity to parasitize beetle grubs (VII). It has not become attenuated *in vitro*. This is ascribed to two facts. First, the kidney tissue culture is evidently adequate in its biological potentialities for this species. No selective environmental factors at work on the worm populations developing in them have been observed. Thus this type of axenic microcosm appears to be a fair sample substitute of the universe in which this nematode species can get along. This alone is of considerable biological interest. Second, the inauguration of each stock culture has been with a sufficient number of worms (100-200) to maintain genetic heterogeneity of the culture strain. Selection of strains apparently has not had an easy opportunity to occur, and a substantially wild population has been maintained.

## IX. SUMMARY

A method of culturing the parasitic nematode, *Neoaplectana glaseri*, axenically in fluid media is described. The parasite develops and multiplies through more than one generation in acid veal or beef heart infusion broth supplemented with a raw liver extract (RLE) that has been prepared without heat, acidified, and sterilized by Seitz filtration. Shaking the cultures in the dark produces larger yields. Other factors bearing on successful cultivation are considered.

The stock culture strain, isolated from a Japanese beetle grub and rendered bacteria-free in 1944, has been carried serially on tissue (usually kidney) on dextrose agar slants. It has retained infectivity for its host.

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CERCARIA MILFORDENSIS NOV. SP., A MICROCERCOUS TREMA-  
TODE LARVA FROM THE MARINE BIVALVE, *MYTILUS*  
*EDULIS* L. WITH SPECIAL REFERENCE TO ITS  
EFFECT ON THE HOST

JOSEPH R. UZMANN

U. S. Fish and Wildlife Service, Milford, Connecticut

*Cercaria milfordensis* was found by the writer in April, 1951 in 4 of 93 (4.3%) intertidal specimens of the common mussel, *Mytilus edulis*, from Mill Neck, Long Island, New York. Subsequently, this larva was found in *Mytilus* from Milford and Bridgeport, Connecticut, in both intertidal and subtidal areas where 30 of 454 (6.6%) mussels collected in 1951 and 44 of 567 (7.7%) in 1952 were found to be infected. Consultation with Dr. V. L. Loosanoff, Director of the U. S. Shellfish Laboratory at Milford, revealed that in 1936, he also had observed the parasite in *M. edulis* from Milford Harbor. Dr. Loosanoff kindly made available a collection of slides of adult *Mytilus* gonad tissue collected at biweekly intervals during the period June 1936–June 1937. Examination of this material showed that 12 of 174 (6.9%) specimens of *Mytilus* were infected with the microcercous larvae.

Previous records of cercariasis in *Mytilus* spp. are European in origin and pertain to the larvae of three trematode species. Dubois (1901, 1903, 1907, 1909) described larval stages of *Gymnophallus margaritarum* [= *Distomum margaritarum* Dubois, 1901] from *M. edulis* and *M. gallo-provincialis*. The metacercaria of this species has held particular interest as a cause of pearl formation in *Mytilus* and other bivalve species, and many conflicting accounts were rendered concerning its biology and identity. Palombi (1924) re-studied the species in *M. gallo-provincialis* in an attempt to clarify the conflicting opinions of the time and concluded that the "pearl trematodes" of Jameson (1902) and later authors were identical with the species of Dubois. Cole (1935) described two cercarial forms, *Cercaria tenuans* and *Bucephalus mytili*, from *M. edulis* in British waters; infection with the former species was termed "orange-sickness" from the orange color imparted to the mantle lobes of infected mussels by masses of orange-pigmented sporocysts. A similar gross appearance is characteristic of mussels heavily infected with the trematode larvae discussed here.

The specific name *milfordensis* is proposed for this larval distome in consideration of the evidence of consistent and long-standing endemic frequency in this locality. It is noteworthy, perhaps, that this parasite has not been reported before from higher latitudes where *M. edulis* has been studied for many years. The writer had previously examined over two thousand specimens of *M. edulis* from Newburyport and Gloucester, Massachusetts, without finding this trematode parasite. It should be noted that the endemic areas cited are quite localized with respect to each other. The Connecticut localities are in close proximity and separated from the New York area only by the north-south reaches of Long Island Sound. Ecologically, the intertidal collecting areas are practically identical, the Sound between them notwithstanding.

The following description is based on a study of naturally emerged cercariae, both living and preserved, and on cercariae within the sporocysts. Permanent preparations of sporocysts and cercariae killed in hot formalin, secondarily fixed in Dubosc-Brasil fluid, and stained with borax-carmin were especially useful for morphological details and measurements which, except where noted, are from permanent preparations of cercariae within sporocysts. Such measurements are considered more accurate than those obtained by other methods. The opacity of living, or unstained, uncleared larvae prevents satisfactory measurement of organs without excessive coverslip pressure and resultant exaggeration of size. Standard histological procedures were followed for a detailed study of the host-parasite relationships. Several combinations of fixatives and stains were employed, the most satisfactory being a Zenker acetic—Iron hematoxylin schedule.

*Cercaria milfordensis* n. sp.

(Figs. 1-2. All measurements in millimeters)

The cercariae are small, stout-bodied, microcercous distomes produced in simple, saccate, motile, orange-pigmented sporocysts in the gonad and digestive gland of *M. edulis*. One hundred ripe sporocysts measured from 0.857 to 1.652 in length with a mean value of 1.246. The sporocysts occur primarily in the blood sinuses and interfollicular lymph spaces of the mantle gonad where infection leads to suppression of gametogenesis or complete atrophy of the reproductive organs. The cercarial bearing sporocyst generations are preceded by, and also accompanied by, smaller, non-pigmented sporocysts which have not been observed to contain other than undifferentiated germ balls. Whether this is a distinct sporocyst generation, or a younger stage of the cercarial generation, remains to be determined. In any event, these antecedent stages are dominant from July to October after which they rapidly give way to cercarial generations which persist and dominate in numbers to a period of maximum development in April, May, and June. In subtidal infections, maturation of cercariae may be attained even later, presumably as a direct result of the lower prevailing temperature. Mature infections with sporocysts containing more than one hundred cercariae were found in several specimens of *Mytilus* collected July 3, 1952 at Bridgeport, Conn. in 10 feet mean low water.

Fully developed, spontaneously emerging cercariae are pyriform, oval or elongate in outline depending upon the degree of contraction. The observed size range of a series of such living larvae was from 0.147 to 0.525. The dimensions of a series of fixed, stained, and mounted larvae were 0.221 to 0.284 in length, by 0.072 to 0.084 in width, with mean values of 0.254 by 0.075.

The oral sucker is relatively large, oval to spherical, usually longer than broad, with the oral opening subterminal in position; it varies from 0.040 by 0.038 to 0.045 by 0.041 with mean values of 0.043 by 0.038. The short prepharynx is 0.002 to 0.004 in length. A subspherical pharynx averaging 0.020 by 0.024 is followed by a short, hyaline esophagus joined by broad digestive ceca that extend to a level slightly posterior to the bifurcation of the excretory vesicle. The walls of the ceca are composed of large polygonal cells which are readily demonstrated with neutral red.

The acetabulum is spherical, slightly larger than the oral sucker, and from 0.045 to 0.051 in diameter with a mean value of 0.048. The approximate sucker-pharynx-acetabulum ratio is 2:1:2.

A small spherical tail measuring only 0.010 to 0.013 in diameter is characteristic of this larva. It is weakly attached to a slight posterior invagination of the body and apparently is without useful function; it may be either lost while the cercariae are still confined to the sporocyst, or retained for several days after emergence.

The excretory vesicle is Y-shaped with the stem shorter than the arms which extend anteriorly and terminate at the esophageal level where they receive the primary tubules. Details of the excretory system were obscured by abundant unicellular glands. Some of the larger of these are figured to show their irregular distribution. Three pairs of cephalic gland ducts emanate from the post-pharyngeal region and terminate in six separate pores on the dorsal lip of the oral sucker. The

cephalic gland cells could not be located with any certainty in either living specimens or in permanent preparations. Some of the gland cells figured probably are cephalic glands, while the others are cystogenous in function.

Survival experiments were conducted with emerged cercariae in filtered sea water at 21.0°, 14.0°, and 7.0° C.  $\pm 1.0^\circ$  C. At 21°, the larvae survived only 24 to 48 hours, whereas at 14°, little or no mortality occurred in replicates until the fourth day and all larvae were dead or moribund by the seventh day. At 7°, five groups of fifteen larvae each were maintained without mortality for seven days while after twelve days, twenty-nine of the original seventy-five larvae were still active. While it has not been established that spontaneous emergence of cercariae occurs more frequently at any particular temperature, it is important to note that maturation of cercarial generations occurs during late winter and spring when the annual temperature cycle is at its lowest (2°–8° C.).

The behavior of *C. milfordensis* offers little direct information bearing on the life cycle. The larvae do not manifest any pronounced taxes, and their role in completing the life cycle appears to be decidedly passive in contrast to the host seeking efforts of many other cercariae. The only activity after emergence consists of periodic contraction and elongation which effects a serpentine but random progress upon the substratum. The suckers, though powerful and well developed, are seldom used in this progress. The survival experiments suggest that increased longevity outside the molluscan host may compensate for the lack of natatory ability.

#### HOST-PARASITE RELATIONSHIPS

*C. milfordensis* is primarily a parasite of the blood vascular system of its host, *M. edulis*. Sporocyst development and asexual multiplication are particularly confined to three mutually related elements of the venous system serving the mantle region. In ascending order of importance, these main groups are: (1) the paired *longitudinal veins* which are central collecting sinuses for blood from all parts of the body; (2) the *external plicate canals*, accessory respiratory lymph channels which transfer venous blood from the greater part of the mantle to the longitudinal veins; (3) the paired *horizontal veins* of the mantle which receive blood from the many ascending pallial veins of that organ and deliver it through the external plicate canals to the longitudinal veins. Among these elements of the vascular system, the horizontal veins are primary foci of infection.

Beginning with the early development of definitive sporocyst generations in late fall, there is a pronounced accumulation of immature and rapidly developing sporocysts within the horizontal veins which course along the entire length of the dorsal level of the mantle slightly below the angle formed by the junction of the mantle and the gill filaments. Eventually the veins become dilated and tightly packed with sporocysts (Fig. 5) which continue to multiply and grow, forcing their way downward and into the mantle through the ascending pallial veins. The vascular system of the mantle eventually loses its organized continuity through rupture of the lymph bearing vessels and appears finally as a more or less continuous hemocoel with sporocysts pervading throughout.

The pathological consequences following this infection are both mechanical and physiological. The "blocking layer"<sup>1</sup> of sporocysts developing in the horizontal

<sup>1</sup> The "blocking layer" of Rees (1936), as originally described, referred to inactive, non-migratory sporocysts which multiplied and remained in a tightly compacted, circumscribed location. The expression is fitting, nevertheless, for the sporocysts of *C. milfordensis*.

veins of the mantle causes a serious reduction in the efficiency of the entire circulatory system. The more important effect, however, is the impingement upon the circulation of the mantle itself, which is destined, seasonally, to become the major seat of gametogenesis. Unfortunately for the host, sporocyst development coincides with the period of annual gonadal development. In addition to these more obvious considerations, the potentially toxic effects of the larval metabolites must be suffered by the host.

The reproductive system of *M. edulis* consists of a racemose complex of genital ducts and follicles which ramify throughout the visceral mass and the mantle lobes. The greater part of the system, however, occupies the mantle lobes which in normal individuals are distended with reproductive tissue prior to spawning (Figs. 3, 4). Although *M. edulis* is normally dioecious, hermaphroditism may occur rarely. During the course of this study, one instance of bisexuality was observed. At the time of sexual ripeness (April, May, and June in these waters) the sexes may be distinguished by the color of the gonadal mass in the mantle; the ripe male gonad appears whitish, while the female follicles with included ova impart a coral to orange color to the mantle. Mussels are sexually spent by late July or early August and the follicles and residual sex products undergo the process of resorption. During this resting phase the mussels accumulate reserve nutritive materials preparatory to a new annual sexual cycle commencing in the late fall. Concurrent with the onset of renewed gametogenic activity, the sporocysts of *C. milfordensis* invade and mass in the dorsal region of the mantle gonad. The blocking layer is most characteristic at this time of the year as asexual multiplication of sporocysts leads to early cercarial development. Sporocyst development precludes normal gametogenesis; follicular development, if it has proceeded at all, is seriously impaired. Larval development in infected mussels keeps pace with the gametogenic processes of normal individuals and it appears that the parasite has advantageously chosen its host's most active period of reproductive activity to serve its own needs. Maximum development and maturation of cercarial generations occurs during the spring. Figure 6 shows the appearance of a portion of the mantle gonad of a male mussel with mature sporocysts dominating the organ. Although the specimen represented was collected in early July in subtidal waters, it is typical of the condition prevailing in the spring in mussels from intertidal areas.

The ability of the host to survive infection has not been determined. During the spring of 1952, however, 30 specimens of infected *Mytilus* were isolated and maintained in trays of running sea water in the laboratory and all but two of these died during the summer. While controls were not kept, it is assumed that the mortality was abnormally high since prior observation of healthy mussels held under similar conditions showed them to be very hardy. The two survivors were dissected in late November and gave proof that infection persists beyond one cycle at least. One specimen contained only sporocysts with undifferentiated germ balls, presumably daughter generations, while the other contained these and cercarial generations as well. The severe intensity of the parasitism suggests that *C. milfordensis* infections are probably lethal under temporary or sustained periods of ecological conditions unfavorable to the host.

*C. milfordensis* is tentatively referred to the FELLODISTOMATIDAE, the members of which mature in marine fishes. The proposal is based upon the discovery of

several specimens of unencysted progenetic larvae referable to the genus *Proctoeces* in the visceral mass of three mussels bearing *C. milfordensis* infections. Morphological comparison of the two forms is favorable, and if the apparent relationship truly exists, an abbreviated life cycle may be possible since the larval *Proctoeces* contain many eggs with well developed, motile miracidia. Experimental studies are projected and it is hoped that decisive information can be presented at a later date.

Buttner (1950) presented a comprehensive review of progenesis in the DIGENEA and listed 34 progenetic species representing 12 families. The present study adds a new family and genus to those compiled by Buttner and provides further evidence that progenesis may be more common than has been suspected.

I wish to express my sincere appreciation to Dr. Horace W. Stunkard, of New York University, for generous counsel. Acknowledgment is due to J. W. Lownsbury for technical assistance and to C. A. Nomejko for preparation of the photomicrographic figures.

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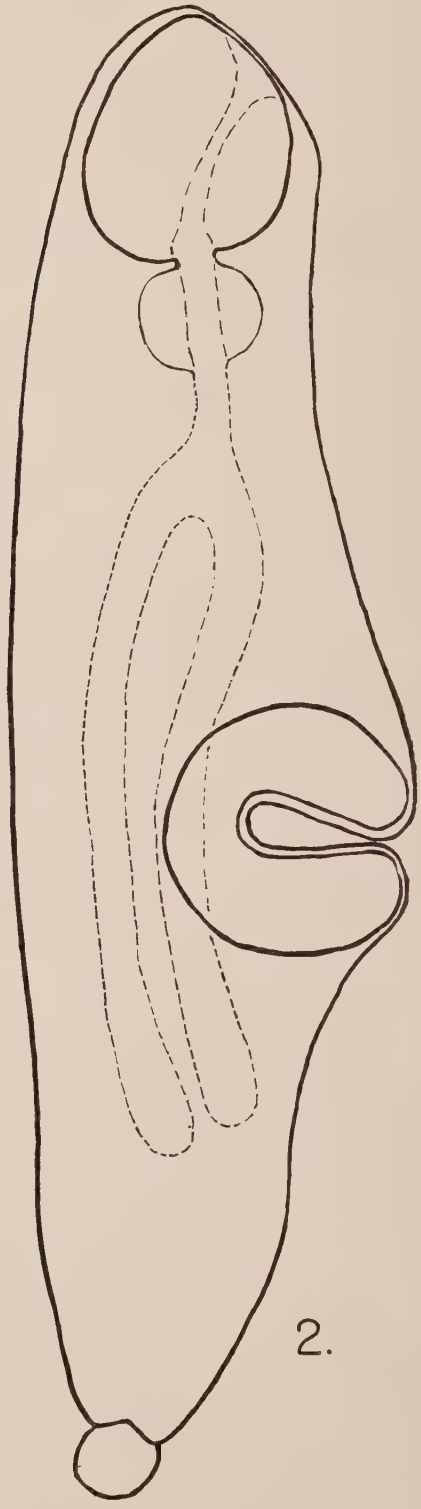
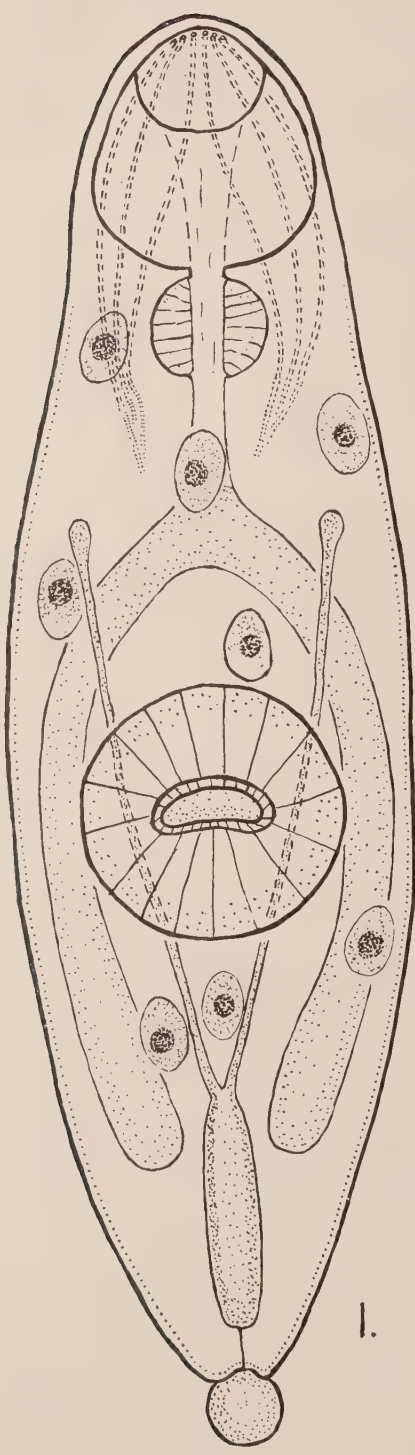
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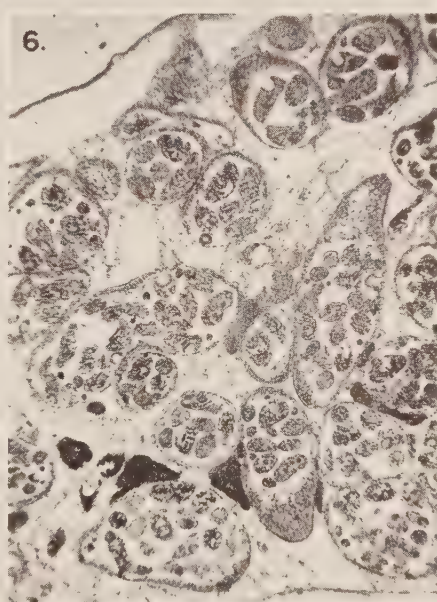
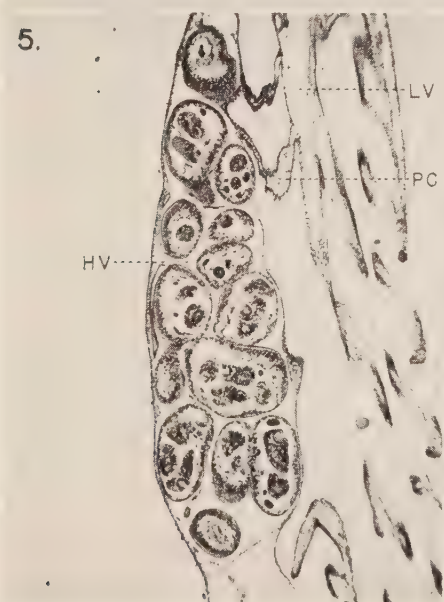
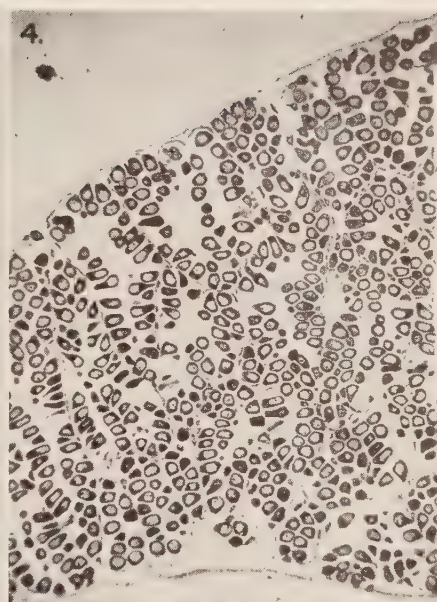
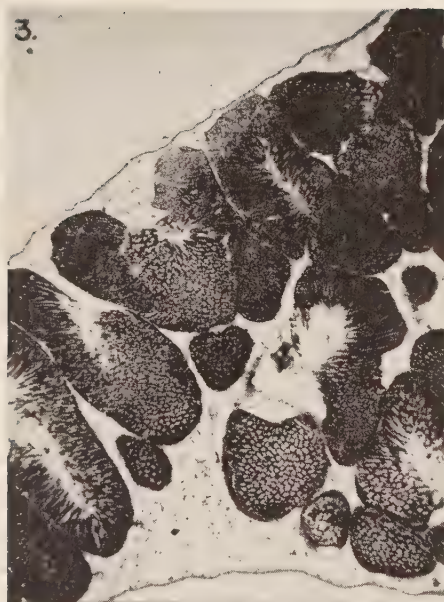
EXPLANATION OF FIGURES  
PLATE I

FIG. 1. *Cercaria milfordensis*, ventral view. Camera lucida outline drawing with freehand detail.

FIG. 2. *C. milfordensis*, lateral view. Camera lucida outline drawing.

PLATE I





## PLATE II

FIG. 3. *Mytilus edulis*, male gonad. Photomicrograph of transverse section through dorsal portion of mantle gonad at mid-body level. Typical appearance of mature follicular complex. Note cords of spermatozoa extending into lumina of follicles. Preserved in April.

FIG. 4. *M. edulis*, female gonad. Plane of section same as Fig. 3. Typical ripe female preserved in April showing follicular arrangement and undischarged ova.

FIG. 5. Parasitized *M. edulis*, sex indeterminate. Section through dorsal level of mantle and gills near point of junction showing early formation of blocking layer of *C. milfordensis* sporocysts within horizontal vein (HV). LV, longitudinal vein; PC, external plicate canal. Preserved in November.

FIG. 6. Parasitized *M. edulis*, male. Plane of section same as Figs. 3 and 4. Typical appearance of heavily parasitized gonad with mature sporocysts dominating entire organ. Deeply stained areas are persisting remnants of atrophied male follicles. Compare with Fig. 3. Preserved in early July.

## THE OCCURRENCE OF PHANEROZOITES OF *PLASMODIUM* *LOPHURAE* IN BLOOD-INOCULATED TURKEYS\*

MIGUEL MANRESA, JR.

Phanerozoites of *Plasmodium lophurae* were first found in brain smears of two 46-day-old turkeys which succumbed on the twenty-first day of infection after the parasitemia in each bird had declined to less than three per cent (Becker and Manresa, 1950). Further studies have been conducted to learn more about the location and time of occurrence of these stages in the tissues of not only young turkeys that succumbed, but also birds sacrificed prior to that eventuality.

### PROCEDURE

The turkeys in which phanerozoites occurred were of the bronze, broad-breasted type. Twenty-five White Hollands were also infected, but in none of them could phanerozoites be found as sacrificed from the fifteenth to the thirty-first day of the infection, nor did any of them succumb to malaria. The claim is not made on the basis of this evidence, however, that phanerozoites cannot develop in white turkeys. The ration was a commercial chick starter without supplement of antibiotics. The strain of *Plasmodium lophurae* employed had been maintained in ducks for two years, but it was passed twice through turkeys, each passage with a duration of six days, before injection into the experimental poults. Each of the latter was injected with  $2 \times 10^8$  parasitized turkey erythrocytes per 100 g. of body weight.

Smears of brain, spleen, liver, kidney, heart and lung by the streak method were fixed in methyl alcohol and stained in Giemsa. Microscopic examinations were made with low, high-dry and oil immersion lenses.

For lack of a better measurement of the intensity of exoerythrocytic infection, a standard called the EE index was devised. One hundred lengths of blood vessels traversing the field of the oil immersion (97 $\times$ ) lens were counted successively, and the total number of schizonts in these 100 lengths recorded to represent the intensity of exoerythrocytic infection. Haas, Wilcox, Laird, Ewing and Coleman (1948) employed mortality of chicks as the criterion of intensity of exoerythrocytic population in *P. gallinaceum* malaria. Since there is a possibility that some of the turkeys with exoerythrocytic forms in the brain would have recovered, the method used here undoubtedly gave more accurate results.

The plasma used in testing the effect of immune plasma on the phanerozoites was collected from recovered turkeys and ducks. About 0.8 cc. per 100 g. of poult body weight was injected intravenously about an hour before injection with the infected cells, and again after 1, 2, 3, 4 and 5 days.

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\* Drawn from doctoral thesis No. 1228 entitled *The Turkey as a Host for Plasmodium lophurae* Coggeshall, 1938, two bound copies of which have been deposited in the Iowa State College library. The research was supported (in part) by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

## RESULTS

It should be stated first that phanerozoites were seen only in brain smears, for smears of spleen, liver, kidney, heart and lung were consistently microscopically negative.

To determine the time during the infection when phanerozoites were most abundant in the brain and to find the range of the time over which they appeared after infection, smears were made of all birds that died of the infection and also of birds killed at various time intervals after infection. A total of 88 turkeys were examined for exoerythrocytic stages (Table 1).

TABLE 1.—*Incidence and Density of Phanerozoites in Brain Smears of 88 Six- to 32-Day-Old Turkeys*

Day after infection	No. of turkeys examined	Result of EE examination		
		No. negative	No. positive	EE index (average)
6	7	7		
7	6	6		
8	2	2		
9	2	2		
10	2	2		
11	2	2		
12	3	3		
13	2	2		
14	2	2		
15	4	2	2	76
16	2	2	0	0
17	5	2	3	128
18	5	3	2	235
19	7	1	6	358
20	7	0	7	239
21	5	1	4	214
22	4	3	1	118
24	4	3	1	102
25	2	2		
26	2	2		
27	2	2		
28	2	2		
29	2	2		
30	2	2		
31	2	2		
41	2	2		
97	1	1		

As shown in Table 1, phanerozoites occurred in the brain from the 15th to the 24th days after infection, by which time the erythrocytic infection had become either negative or nearly so. Of the 43 poultz sacrificed during that interval 26, or 60.5 per cent, were positive. Data are mixed from those that died or were killed. The greatest density occurred on the 18th, 19th, 20th, and 21st days after infection, as shown by the EE indices of 235, 358, 239, and 214, respectively, and the numbers of turkeys that were positive for exoerythrocytic forms.

Similar observations on *P. gallinaceum* by Haas, Wilcox, Davis and Ewing (1946) showed cases in which the exoerythrocytic forms predominated and death due to them occurred about 11 days after inoculation.

James and Tate (1938), in reporting the exoerythrocytic forms of *Plasmodium gallinaceum*, stated that there was great irregularity in the period of the infection during which exoerythrocytic stages developed. The results of the present study on exoerythrocytic stages showed that the cycle occurred from about the 15th to the 24th day, and after parasitemia had practically cleared up. This observation is in agreement with the results reported by Haas *et al.* (1948) after their study of the different responses of exoerythrocytic forms to alteration in the life-cycle of *Plasmodium gallinaceum*. They found 3 general patterns, the second of which

occurred in chicks infected by blood-inoculation. In it there was an acute stage, marked by heavy erythrocytic infection. Many chicks died during this period, but exoerythrocytic forms became prevalent about the third week after inoculation in those that survived.

Data were obtained from microscopic examinations of smears stained for exoerythrocytic forms from turkeys receiving immune duck plasma, immune turkey plasma, and physiological salt solution (the controls). The smears were made at intervals of a number of days after inoculation, or at the time of death. The birds were sexed at post-mortem. Exoerythrocytic forms when present were rated in intensity by the EE index previously described. Results of the study appear in Table 2.

TABLE 2.—*Effect of Sex, Plasma, and Parasitemia on Exoerythrocytic Forms.*  
(EE = Exoerythrocytic; PM = Post Mortem)

Day after infection	Turkey		% Parasitized cell		Result of EE examination					
					Duck plasma-recipients		Controls		Turkey plasma-recipients	
	No.	Sex	At peak	On day of EE exam.	Negative	Positive (EE index)	Negative	Positive (EE index)	Negative	Positive (EE index)
12	22	M	55.0	0.1						
12	32	M	61.0	0.2						
12	94	F	58.0	0.2						
17	00	M	65.0	3.6		290				
17	28	F	49.0	0.0						35
17	96	F	51.0	0.3				52		
18	98	F	48.0	1.0				240PM		
19	21	F	64.0	3.3		310				
19	30	F	31.0	0.1						210
19	95	M	46.0	0.1				150		
19	97	F	46.0	0.1				222		
20	23	M	45.0	5.4		284PM				
20	29	M	18.5	4.6						325PM
20	31	F	24.5	1.7						263PM
20	24	M	60.0	1.3		161				
20	27	F	43.5	0.0						276
21	90	F	42.5	0.1				228		
22	25	M	48.0	3.6		118				
30	99	F	31.8	0.5			..			

Of the 19 turkeys studied eight were males and 11 were females. Fifteen, of which six were males and nine were females, showed phanerozoites in the brain smears (Table 2). The data indicate that sex did not have a perceptible effect on exoerythrocytic formation.

That duck or turkey plasma did not influence exoerythrocytic formation is shown by the fact that five turkeys in each of the duck plasma-recipients, turkey plasma-recipients, and controls were positive for phanerozoites. The EE indices for the 15 positive turkeys varied in intensity since they were taken at various intervals after inoculation. The EE indices are presented here merely to show the relative intensity of exoerythrocytic infection in the groups at various days after inoculation.

It will also be noted that all the turkeys tested for phanerozoites had been cleared of parasitemia, or nearly so, at the time they died of the exoerythrocytic infection or were killed for brain smears. This result is also in full accord with that reported by Huff and Coulston (1946) in their work with *Plasmodium gallinaceum* in chickens, wherein they found that when malaria was produced by the inoculation of parasitized erythrocytes, phanerozoites appeared late in the infection, usually reaching a maximum in numbers during the third or fourth week.

## CONCLUSIONS

1. Phanerozoites of *Plasmodium lophurae* occur in the brain of turkeys from the fifteenth to the twenty-fourth days after inoculation with infected erythrocytes. The greatest concentration of exoerythrocytic stages in the brain occurs around the nineteenth day.
2. The phanerozoites occur in poult after they have practically recovered from the parasitemia, when the incidence of infected cells ranges from 0.0–5.4 per cent.
3. Sex, immune plasma and intensity of the parasitemia apparently do not influence the development of exoerythrocytic stages.

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## TWO NEW HYSTRICHOPSYLLID FLEAS FROM JAPAN (SIPHONAPTERA: HYSTRICHOPSYLLIDAE)<sup>1</sup>

E. W. JAMESON, JR.<sup>2</sup> AND NOBUO KUMADA<sup>3</sup>

These two new species of fleas are widespread and occur on some of the most common small mammals in Japan. One of them parasitizes wild murid rodents, and because these rodents may live in and about human dwellings, their external parasites are potential vectors of human diseases.

### PALAEOPSYLLA NIPPON, NEW SPECIES

The genus *Palaeopsylla* is typically a parasite of small insectivores (Soricoidae) of the Palaearctic Region, and this is the first record for *Palaeopsylla* from Japan. *Palaeopsylla nippon* is most closely related to *P. remota* Jordan, 1929, a species which occurs to the east and south of Japan.

*Male*: Chaetotaxy of head as illustrated (Pl. 1, A). The long, prominent setae are constant, but the minute, more or less scattered setae vary slightly in number and position. Five long preantennal setae: three above the eye and two larger setae below (or in front of) the eye. Eight long postantennal setae: a diagonal (anterior) row of three, a marginal (posterior) row of three, and two long setae on the upper margin of the antennal groove. Antennae with numerous fine setae on the mesal side of the club; second segment of antennae with setae extending for no more than one-third the distance of the club (Pl. 2, A). Frontal tubercle above level of eye, acute, and directed upward. Eye reduced, at the top of the genal ctenidium.

Pronotal ctenidium (Pl. 1, A) of 9–10 spines per side. Cervical sclerite moderate in length, with a ventral concavity. Mesonotum (Pl. 1, D) with one to three pseudosetae per side, beneath the collar; no such pseudosetae on metanotum. No pleural ridge on pleural-sternal plate. Mesepisternum with three or four setae of varying lengths; mesepimerum with three long setae. Metepisternum with one and metepimerum with four long setae.

Apical spinelets on abdominal terga: I, 2 (or 3); II, 2 (1–3); III, 2 (1–3); IV, 2 (or 1); V, 1 (or 2); VI, 0 (or 1). Three antepygidial bristles: middle the longest and upper the shortest.

Fifth tarsal segment on each leg with six pairs of plantar bristles: a basal, ventral pair, four lateral pairs, and apical ventral pair (Pl. 2, C and D). Coxa III with 2–4 long setae, but without an inner patch of spinelets.

Clasper and moveable finger as illustrated (Pl. 2, F and G). Especially characteristic are the rather heavily sclerotized apical angle of the clasper (Pl. 2, G) and the subbasal projection on the anterior margin of the finger (Pl. 2, F). Penis rod directed upward, not completing one turn. Sternite VIII broadly rounded with no lobes, sinuses or marginal setae, but with two or three basal setae.

*Female*: Chaetotaxy essentially as in male, but slightly fewer setae in some regions. Only one long seta on the upper margin of the antennal groove, and anterior seta (of the male) being absent. Second segment of the antenna bearing two long setae on the upper edge (Pl. 2, B); mesal side of club with no setae. Genal ctenidium as in male.

Pronotal ctenidium of 9–10 spines, as in male. Two pseudosetae under collar of mesonotum; none on metanotum. Thorax as in male.

Apical spinelets of abdominal terga slightly fewer than in male: I, 2 (or 3); II, 2 (or 3); III, 2; IV, 1 (or 2); V, 1 (or 0); VI, 0 (or 1). Antepygidial bristles (Pl. 1, B) as in male. Spiracular opening of tergite VIII about half as large as pygidium.

Spermatheca (Pl. 1, C) with head slightly concave ventrally and convex dorsally; tail about as long as head, and curved slightly caudally at apex. Sternite VII (Pl. 1, B and C) with a long but rounded basal lobe, bordered both dorsally and ventrally by a sinus. Anal stylet (Pl. 2, E) with a long straight apical seta and two, short subapical setae. Sternite VIII (Pl. 1, B) digitate, bluntly rounded distally, with four to six apical setae on each side: two apical setae and two subapical, heavier setae. Tergite VIII with about six setae on caudal margin, basally.

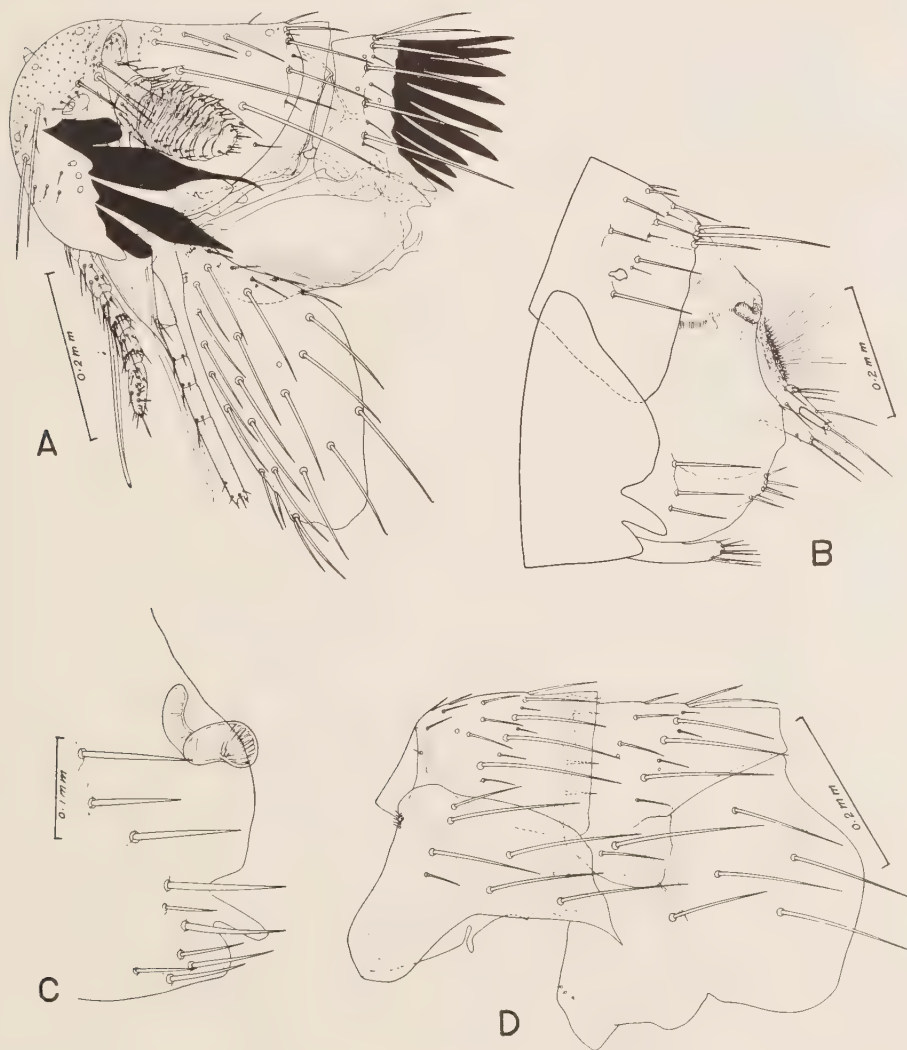
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<sup>2</sup> Department of Zoology, University of California, Davis, California.

<sup>3</sup> Medical Zoological Laboratory, Institute for Infectious Diseases, University of Tokyo, and Medical Zoology Section, Tokyo Medical and Dental University.

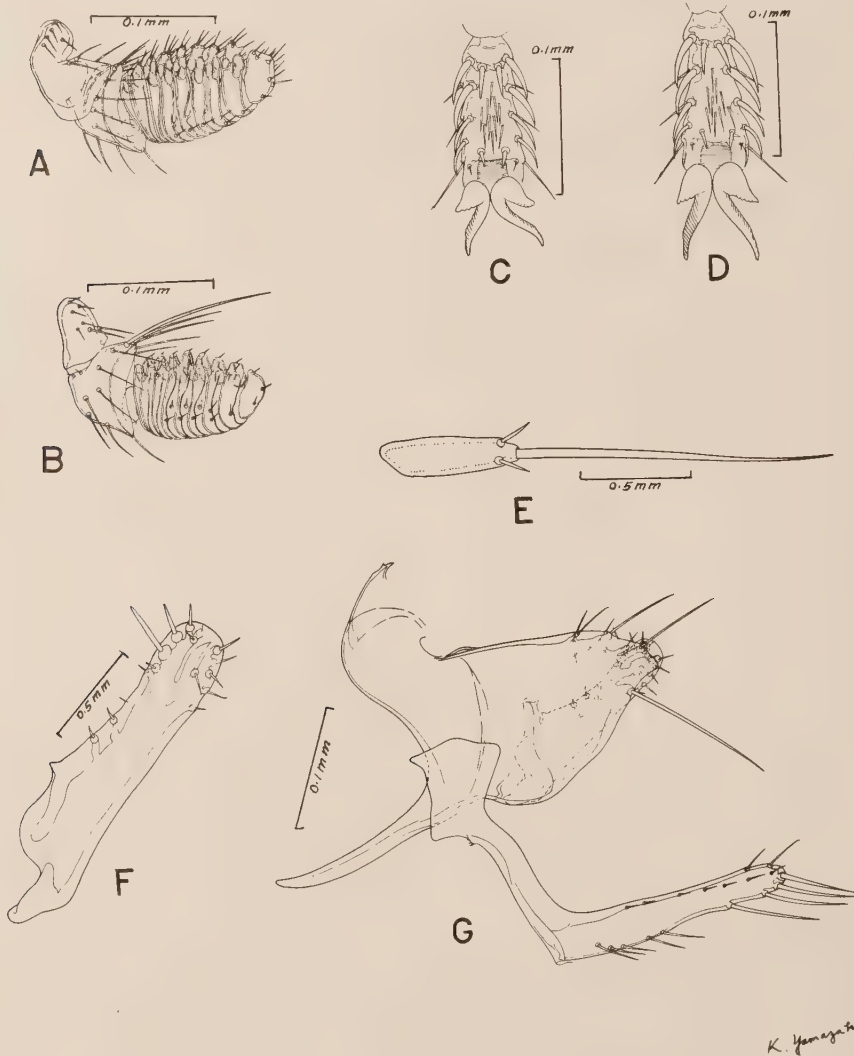
*Types:* Holotype male and allotype female from the shrew-mole, *Urotrichus talpoides* Temminck and Schlegel, 5 miles North of Kyoto, Kyoto Prefecture, Honshu; 7 March 1952. Fifteen males and eight females from the type host and type locality, collected between March and May, designated as paratypes. Holotype, allotype, and two pairs of paratypes deposited in the U. S. National Museum,



PL. 1. *Palacopsylla nippon*, new species. A, head, prothorax, and coxa I of male; B, terminal segments of female; C, spermatheca and sternite VII of female; D, meso- and metathorax of male.

No. 61,834. Paratypes deposited also at Medical Zoological Laboratory, Institute for Infectious Diseases, University of Tokyo; Entomological Laboratory, Kyushu University, Fukuoka; U. S. Army Medical Service Graduate School, Washington, D. C.; Rocky Mountain Laboratory, Hamilton, Montana; Division of Entomology,

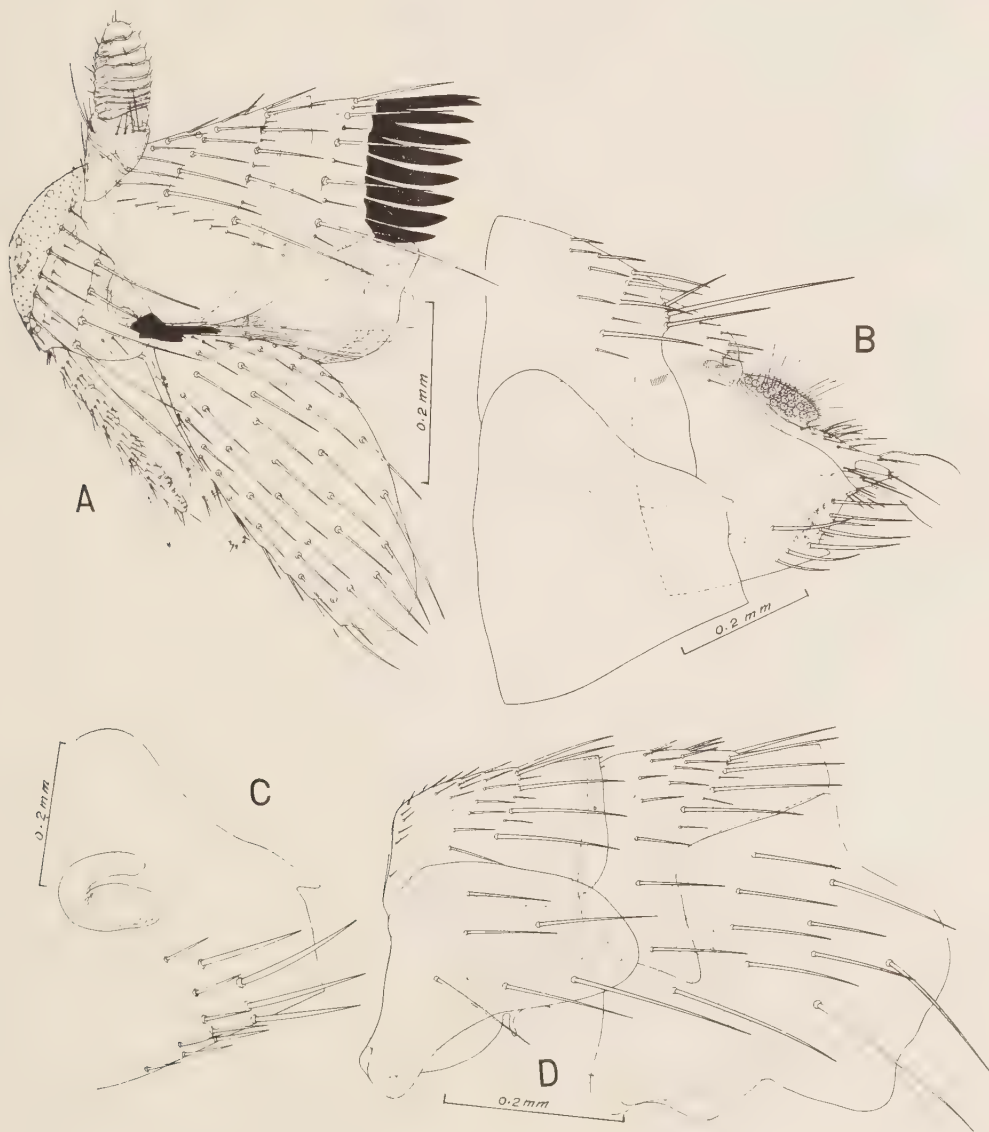
Department of Agriculture, Ottawa, Canada; and the British Museum (Natural History), Tring, Hertfordshire. In addition to the paratype series, specimens of *Palaeopsylla nippon* have also been examined from Shiga, Mie, and Yamanashi Prefectures, collected from *Urotrichus talpoides*; and from Nagano Prefecture from *Dymecodon pilirostris* True, another species of shrew-mole.



PL. 2. *Palaeopsylla nippon*, new species. A, antenna of male; B, antenna of female; C, mesotarsal segment V of male; D, metatarsal segment V of male; E, anal stylet of female; F, moveable finger; G, clasper, moveable finger, and sternite IX.

*Diagnosis:* *Palaeopsylla nippon* is most closely related to *remota* Jordan, 1929, a species known from West China, Sikkim, Burma, and Taiwan. *P. nippon* is distinct in the somewhat angulate, sclerotized apex of the process of the clasper, and in the subbasal projection on the anterior margin of the finger; in *remota* the apex of the process is unsclerotized and rounded, and the anterior margin of the finger

is without a projection. Female of *nippon* has a much longer and narrower lobe and deeper sinuses on sternite VII than *remota*; sternite VIII deeper than in *remota* and with four setae of two sizes.

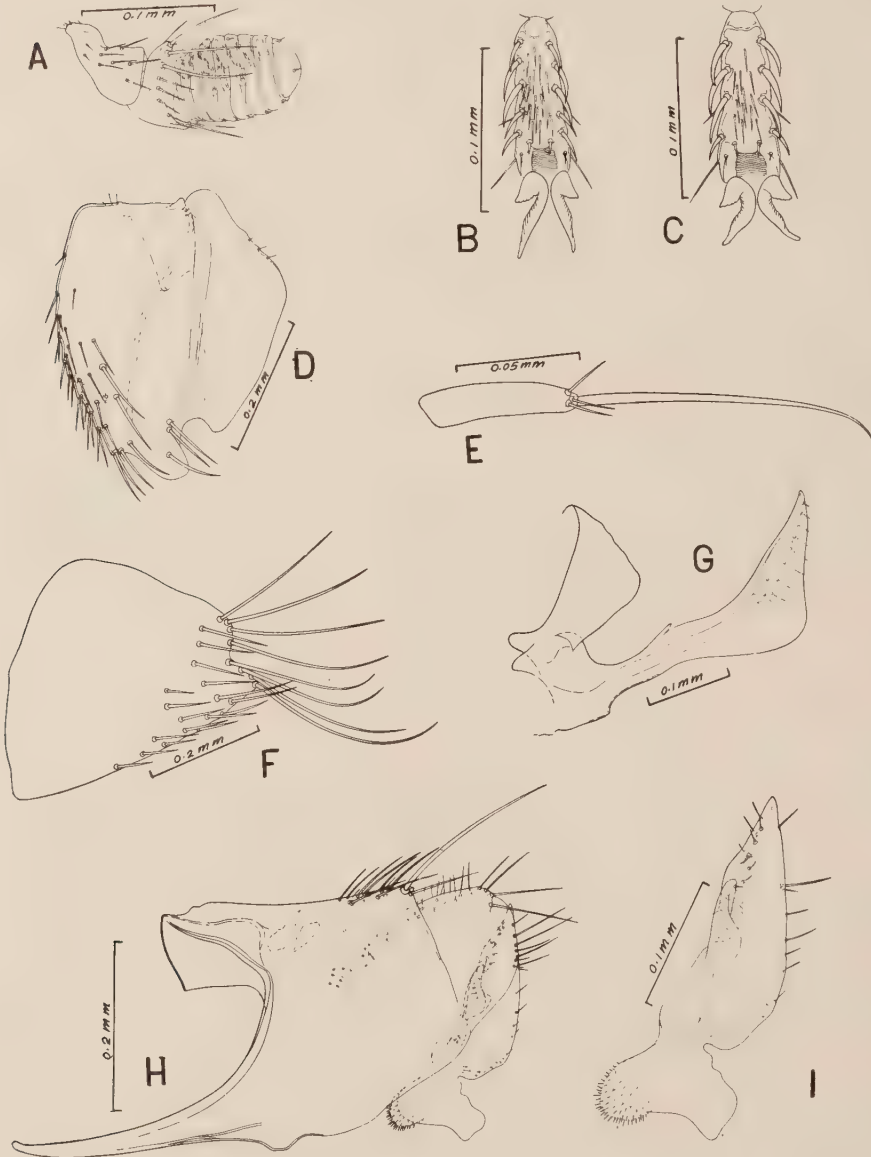


PL. 3. *Neopsylla sasai*, new species. A, head, prothorax, and coxa I of male; B, terminal segments, female; C, spermatheca and sternite VII of female; D, meso- and metathorax of male.

#### NEOPSYLLA SASAI, NEW SPECIES

Various species of the genus *Neopsylla* parasitize murid and sciurid rodents. The genus is Holarctic in distribution, but this is the first record for Japan.

*Male*: Chaetotaxy of head as illustrated (Pl. 3, A). Preantennal region with two rows of long setae: the first row with five long and two short setae, and the second row with four long setae. Between these two rows and closer to the first row is a row of six or seven minute



K. Yamaguchi

PL. 4. *Neopsylla sasai*, new species. A, antenna of female; B, mesotarsal segment V of male; C, metatarsal segment V of male; D, coxa III of male; E, anal stylet of female; F, sternite VIII of male; G, sternite IX of male; H, tergite VIII, clasper, and moveable finger of male; I, moveable finger.

setae. Postantennal region with three rows of setae: five setae in each of the first two rows and five long and four short setae in the third (submarginal) row. About eight short setae on the upper margin of the antennal groove. One long seta on the second segment of the antenna; numerous fine setae on the inner margin of the antennal club. Eye unpigmented.

Genal spines broadly overlapping. Cervical sclerite small and amorphous. Frontal tubercle above level of eye.

Pronotal ctenidium of 9 spines to a side. One or two pseudosetae under collar of mesonotum and none under collar of metanotum. Abdominal terga I–VI each with one apical tooth per side, placed very high. Three antepygial bristles; the middle the longest and the upper the shortest. Spiracular opening on tergite VIII smaller than pygidium.

Hind coxa (Pl. 4, D) with a mesal patch of spiniform setae. Fifth segments of the fore- and mid-tarsi with five lateral plantar bristles; fifth segment of the hindtarsus with four pairs of lateral plantar bristles (Pl. 4, B and C).

Process of clasper (Pl. 4, H) with no sinus; with numerous submarginal setae, and about six scattered setae on the lower vertical margin. Moveable finger (Pl. 3, I) with the sides parallel for the lower two thirds, converging at the apical third; about twenty slender setae on the lower angle; with a membranous appendage ventrally. Distal arm of sternite IX (Pl. 4, G) bent slightly anteriorly at about the mid-point; with 10–14 rather delicate setae, but no spiniform setae. Sternite VIII with 10–12 heavy setae on the margin (Pl. 4, F). Tergite VIII (Pl. 4, H) with an angle of about 90 degrees, and a large seta apically.

*Female*: Chaetotaxy similar to that of male. Segment II of antenna (Pl. 4, A) with several setae extending about two-thirds the length of the club.

Pronotal comb with 9 spines to a side. One or two pseudosetae under collar of mesonotum, but none on metanotum. Abdominal apical spinelets as in male, except that tergite VI is usually without a spinelet. Spiracular opening (Pl. 3, B) as in male.

Spermatheca (Pl. 3, C) with head rounded, about twice as long as high; neck not projecting into the head, bent backward dorsally. Sternite VII (Pl. 3, B and C) with a small, acute lobe which is angulate; beneath this projection, sternite VII is rather straight.

*Types*: Holotype male and allotype female from the wood mouse, *Apodemus speciosus* (Temminck and Schlegel), Mt. Hiei, Shiga Prefecture, Honshu; 27 March 1952. One pair of paratypes with the same data as the types. Three males and seven females, designated as paratypes, from Kyoto, Mie, Saitama, and Miyagi Prefectures from the type host and from *Apodemus geisha* (Thomas). Holotype, allotype and one pair of paratypes deposited in the U. S. National Museum No. 61,835. Paratypes deposited also in the Medical Zoological Laboratory, Institute for Infectious Diseases, University of Tokyo; Entomological Laboratory, Kyushu University, Fukuoka; U. S. Army Medical Service Graduate School, Washington, D. C.; Rocky Mountain Laboratory, Hamilton, Montana; Division of Entomology, Department of Agriculture, Ottawa, Canada; and the British Museum (Natural History), Tring, Hertfordshire.

*Diagnosis*: Fixed process of clasper with no sinus, a character found only in *alicna* Jordan and Rothschild, 1911 and *anoma* Rothschild, 1913, both described from China. *N. sasai* possesses three rows of occipital setae whereas *aliena* has only two rows; *anoma* is characterized by having a sclerotic rod at the dorso-apical angle of the aedeagal apodeme, and such a rod is absent in *sasai*. In addition *sasai* possesses a patch of small, spiniform setae on the inner surface of coxa III, and the membranous ventral extension of the finger (clearly seen on dissected specimens) seems to be uncommon. Sternite IX bears a row of fine hairs whereas most allied species of *Neopsylla* have spiniform setae on sternite IX. The female of *sasai* is distinct in the spiniform setae on the inner surface of coxa III, the shape of the spermatheca, and the acute angle of sternite VII. The two or three sinuate setae on the apical part of abdominal segment X are quite characteristic.

It is a pleasure to name this species for Dr. Manabu Sasa, our friend who has done much to foster and encourage the study of medically important insects in Japan.

To Mr. F. G. A. M. Smit we are greatly indebted for his help with the diagnoses of these species. Many of the specimens were collected with the generous cooperation of Dr. Mitosi Tokuda. The illustrations were made by Mr. Kakuzo Yamazaki.

## WILLIAM WALTER CORT

President, American Society of Parasitologists, 1930

It has been the custom of the Society since 1932 to publish the portrait of the retiring president with his presidential address. Dr. W. W. Cort was president of the Society in 1930 before this custom was initiated. Since the Journal has never published his portrait, it is being presented in this issue. It seems appropriate to do so at this time, since Doctor Cort retired June 30, 1953 as Professor and Chairman of the Department of Parasitology of the School of Hygiene and Public Health of The Johns Hopkins University. It is particularly appropriate, at this time, to recognize Doctor Cort's relationship to the American Society of Parasitologists since he was not only one of its founders but he or the members of his staff have served the Society as officers continuously since its founding.

W. W. Cort was one of the small group of the Washington-Baltimore parasitologists in the Helminthological Society of Washington who initiated and developed plans for the formation of the American Society of Parasitologists. The Society was organized in 1924-25 and held its first annual meeting with the American Association for the Advancement of Science at Kansas City Dec. 29-31, 1925 with H. B. Ward as president, C. A. Kofoid as vice-president, and W. W. Cort as secretary-treasurer. There were 321 members at that time of whom more than 60 attended and 38 presented papers on the program. Cort was reelected secretary-treasurer for five years until his election as president for the year 1930. His presidential address on Ascariasis in United States, is an illustration of the epidemiological approach to the study of the parasitic infections, which he so ably developed and fostered.

Cort was elected to the council of the Society (as council member at large) in 1931. During this year, through Cort's leadership, arrangements were completed for the transfer of the Journal of Parasitology by the late H. B. Ward to the Society. Beginning in 1932 with volume 19, the Journal became the property and Official Publication of the American Society of Parasitologists with Cort as Chairman of the Editorial Committee; the other two members of this committee were Robert W. Hegner and Francis M. Root. Cort continued to edit the Journal for 5½ years, through 1937, during which time it developed from a quarterly of 60-80 pages each to a bi-monthly journal of nearly 100 pages each. Cort edited the Journal again in 1948 while H. W. Stunkard the present editor was on sabbatical leave.

In the intervening years Cort has served at various times on the Council of Society, on the Editorial Board of the Journal, as the Society's representative on the Council of the American Association for the Advancement of Science, and as the Society's representative on the Division of Biology and Agriculture of National Research Council. He is currently the Society's representative on the Governing Board of the American Institute of Biological Sciences.

W. W. Cort was born at Leon, Iowa, April 28, 1887, raised in Colorado Springs, Colorado, and educated in Colorado (A.B. Colorado College 1909) and Illinois, where he earned the A.M. (1911) and Ph.D. (1914) degrees at the University of



WILLIAM WALTER CORT

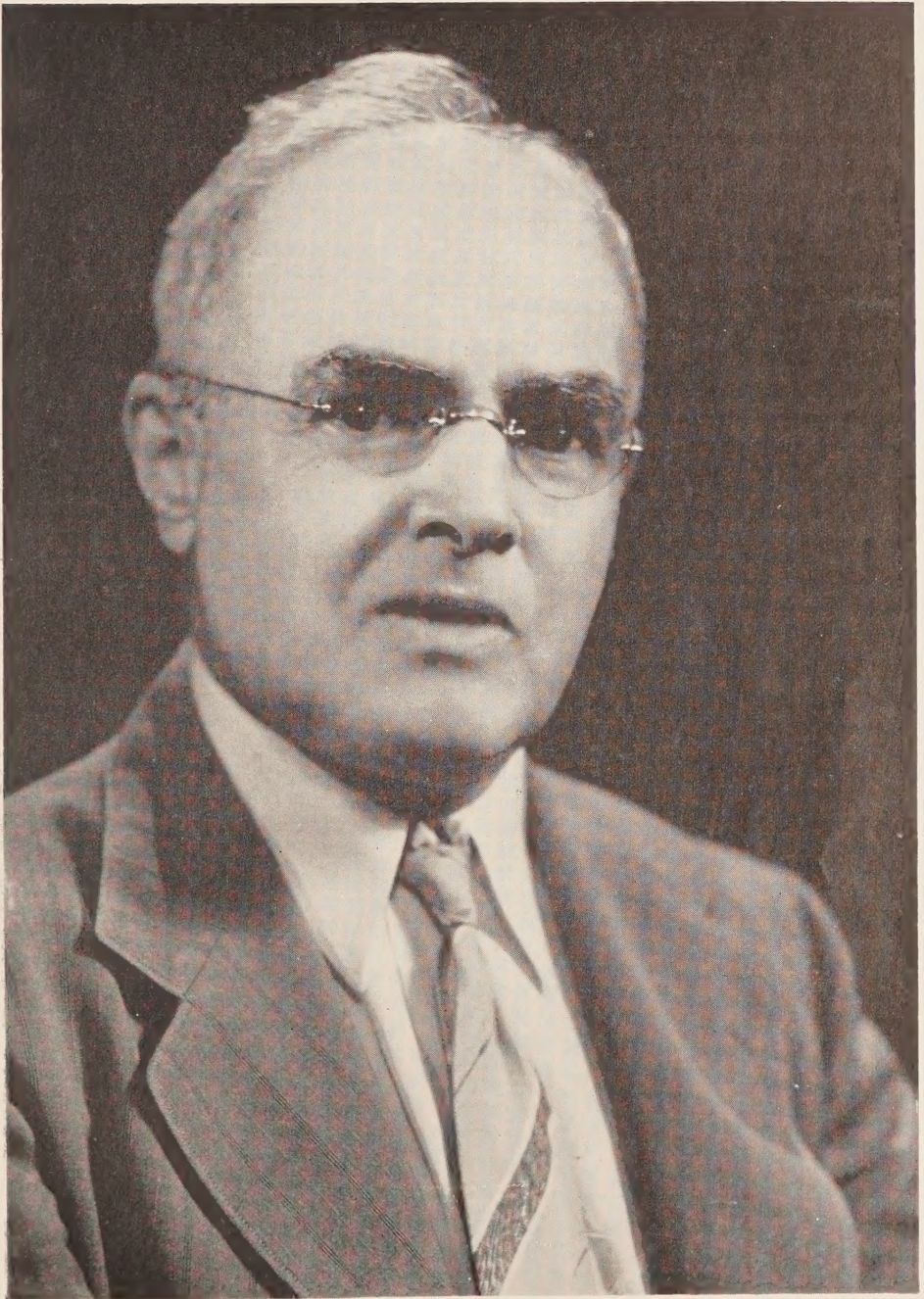
Illinois under the late H. B. Ward. He taught one year at Colorado College (1912-13), two years at Macalester College (1914-16) and three years at the University of California (1916-19), before joining the faculty of the School of Hygiene and Public Health of The Johns Hopkins University. Appointed an Associate in 1919 by the late Robert Hegner, who was then Chairman of the Department (Medical Zoology), he was promoted to Professor in 1925 and became head of a separate department (Helminthology) in 1927. Upon Hegner's untimely death in 1942, Cort became head of the recombined Department which was now designated formally, for the first time, as the Department of Parasitology.

The successful careers of the Doctor of Science graduates of this center of graduate education in Parasitology attest the vision, guidance, and leadership of Hegner, Cort, and Root who developed it. These men, with a succession of junior faculty members, have launched 111 men and women into careers in Science.

Cort has been awarded two honorary degrees, both Doctor of Science; the first by the University of North Carolina in June 1946, and the second by his Alma Mater, Colorado College, in May 1949.

Space does not permit even an abbreviated recital of Cort's professional activities and accomplishments. His 120 odd books, monographs, and papers in scientific journals include the results of two main lines of research. Beginning with his graduate student days and continuing up to the present time at the University of Michigan Biological Station, he and his students have studied the biology of trematodes and in the last two decades have unravelled many of the mysteries of the germ cell cycles of the digenetic trematodes. Beginning with his work on hookworm and ambiasis in California, he and his associates and students have pioneered in the development of field studies on the epidemiology of the parasitic diseases of man and coordinated laboratory studies on immunity to parasitic infections. Particular attention has been given in these studies to the influence of nutritional factors on the immunity to parasitic infections and the resulting effect upon the epidemiology of parasitic diseases.

Although Cort has reached retirement age and has been formally retired by The Johns Hopkins University, he is really not going to retire. He has accepted an appointment as Research Professor of Parasitology at the School of Public Health of the University of North Carolina in the department headed by one of his former students (John Larsh, Sc.D. 1942). He will also continue his studies of the biology of digenetic trematodes during the summers at the University of Michigan Biological Station. Both Dr. and Mrs. Cort continue in excellent health and we can expect to see them regularly at the annual meetings of the Society. They will find time to welcome any of their many friends who visit Chapel Hill, North Carolina.—GILBERT F. OTTO.



WILLIAM A. RILEY

## WILLIAM A. RILEY

President, American Society of Parasitologists, 1931

Dr. W. A. Riley is a charter member of the Society and a member of its original council. He also holds the distinction of being the only person whose name has appeared actively on the cover of every issue of the Journal of Parasitology to date. He was on the Editorial Board when the Journal was founded by H. B. Ward, took a leading part in securing advertising from scientific companies during its early years, and shared in the responsibilities of its acquisition by the Society in 1932. He was one of the three members of the Editorial Committee from 1934 until 1950 when he decided reluctantly he must resign these exacting duties though he remains on the Editorial Board and takes an active interest in the Journal.

On December 31, 1930, Dr. Riley was elected president of the American Society of Parasitologists but he relinquished the privilege of presiding at the December 1931 meeting because of absence during a sabbatical year at Lingnan University in China.

He is Professor Emeritus of Zoology and of Entomology at the University of Minnesota and has been head of each department. As senior author of Riley and Johannsen's *Medical Entomology*, now in its second edition, and as a pioneer teacher in this country of Parasitology, Medical Entomology and Protozoology, he has guided many others into ways of critical and scholarly work. What he has often said in praise of others is particularly true of him, "A scientist is known not only by the caliber of his contributions, but also by the character and accomplishments of those whom he has trained."

His high sense of duty and his long and generous service to the Society and to the Journal are an inspiration to us all.—W. L. JELLISON, C. B. PHILIP and F. G. WALLACE.





*Elery R. Becker*